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Qualitative and quantitative comparison of glucose transport activity and glucose transporter concentration in plasma membranes from basal and insulin-stimulated rat adipose cells

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Conditions are described which allow the isolation of rat adipose-cell plasma membranes retaining a large part of the stimulatory effect of insulin in intact cells. In these membranes, the magnitude of glucose-transport stimulation in response to insulin was compared with the concentration of transporters as measured with the cytochalasin-B-binding assay or by immunoblotting with an antiserum against the human erythrocyte glucose transporter. Further, the substrate- and temperature-dependencies of the basal and insulin-stimulated states were compared. Under carefully controlled homogenization conditions, insulin-treated adipose cells yielded plasma membranes with a glucose transport activity 10–15-fold higher than that in membranes from basal cells. Insulin increased the transport $V_{max.}$ (from 1400 ± 300 to 15300 ± 3400 pmol/s per mg of protein; means $\pm$ S.E.M.; assayed at 22 °C) without any significant change in K_m (from 17.8 ± 4.4 to 18.9 ± 1.4 mM). Arrenhius plots of plasma-membrane transport exhibited a break at 21 °C, with a higher activation energy over the lower temperature range. The activation energy over the higher temperature range was significantly lower in membranes from basal than from insulin-stimulated cells [27.7 ± 5.0 kJ/mol (6.6 ± 1.2 kcal/mol) and 45.3 ± 2.1 kJ/mol (10.8 ± 0.5 kcal/mol) respectively], giving rise to a larger relative response to insulin when transport was assayed at 37 °C as compared with 22 °C. The stimulation of transport activity at 22 °C was fully accounted for by an increase in the concentration of transporters measured by cytochalasin B binding, if a 5% contamination of plasma membranes with low-density microsomes was assumed. However, this 10-fold stimulation of transport activity contrasted with an only 2-fold increase in transporter immunoreactivity in membranes from insulin-stimulated cells. These data suggest that, in addition to stimulating the translocation of glucose transporters to the plasma membrane, insulin appears to induce a structural or conformational change in the transporter, manifested in an altered activation energy for plasma-membrane transport and possibly in an altered immunoreactivity as assessed by Western blotting.

INTRODUCTION

The stimulatory effect of insulin on glucose transport in the adipose cell persists after homogenization of cells and can be demonstrated in an isolated plasma-membrane preparation (Martin & Carter, 1970). This so-called 'fossil effect' has facilitated the study of the mechanism of insulin action and has ultimately provided two crucial findings: (1) that adipose cells contain a large intracellular pool of glucose transporters, from which transporters are translocated to the plasma membrane when cells are exposed to insulin (Wardzala *et al.*, 1978; Suzuki & Kono, 1980; Cushman & Wardzala, 1980; Wheeler *et al.*, 1982); and (2) that the qualitative characteristics of glucose transport with respect to temperature-dependence and K_m were indistinguishable for plasma membranes from basal and insulin-stimulated adipose cells (Ludvigsen & Jarett, 1980). Thus, in contrast with the activation of pre-existing transporters, as had been suggested previously (Carter-Su & Czech, 1980), an increase in the concentration of transporters appeared to account for the stimulatory effect of insulin.

In spite of the substantial evidence that insulin triggers the insertion of intracellularly stored glucose transporters into the plasma membrane, two lines of evidence have led to the suggestion that the hormone might produce an additional activation of transporters. Firstly, only a portion of the effect of insulin was retained in membranes from cells exhibiting a large response to the hormone. In cells, insulin can stimulate the transport rate up to 30-fold, whereas the effects on transport and transporter concentrations retained in the plasma membranes were only 2–5-fold (Martin & Carter, 1970; Ludvigsen & Jarett, 1980; Simpson *et al.*, 1983). Secondly, a decrease in the transport K_m has been reported in highly insulin-responsive adipose cells (Whitesell & Abumrad, 1985, 1986; Toyoda *et al.*, 1987). This finding, though not generally accepted (Martz *et al.*, 1986), appeared to confirm that insulin may change transporter function as well as number. In addition, it has been shown that adenylate cyclase modulators regulate glucose transport activity in adipose cells by altering the transporter intrinsic activity (Joost *et al.*, 1986).

In order to assess the possibility of an additional effect

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of insulin on glucose-transporter intrinsic activity, the present study describes a thorough quantitative comparison of transport activity with transporter concentration in plasma membranes from highly insulin-responsive adipose cells. Conditions are described under which a large part of insulin action on glucose transport in the intact cell can be retained in the plasma-membrane preparation. In these membranes, which retain a greater portion of the response to insulin than does any preparation described previously, glucose transport activities in the basal and insulin-stimulated states are compared with transporter concentration and characterized with respect to temperature- and substrate-dependency. The results show that translocation of transporters can account for at least a 10-fold increase in glucose-transport $V_{max.}$ in the plasma membranes from insulin-stimulated cells. In addition, however, insulin appears to change the activation energy of transport, giving rise to an overall 15-fold stimulation of transport activity observed in the plasma membranes. These results suggest that insulin produces an additional structural or conformational modification of the translocated glucose transporter.

MATERIALS AND METHODS

Animals and cell preparation

Male rats (CD strain; Charles River Breeding Laboratories), weighing 170–200 g, were used throughout. The rats were anaesthetized with CO_2 and killed by decapitation between 08:00 and 09:00 h, and the epididymal fat-pads were removed. The adipose tissue was minced and digested with collagenase (Type 1; Cooper Biochemical) as described previously (Rodbell, 1964). All incubations were carried out in a Krebs–Ringer Hepes buffer, pH 7.4, containing 10 mM-$NaHCO_3$ and 30 mM-Hepes (Karnieli *et al.*, 1981). The buffer contained 2.5 mM-glucose, 200 nM-adenosine and 5% (w/v) bovine serum albumin (fraction V; Reheis Chemical Co.). These conditions were originally developed by Honnor *et al.* (1985) to decrease fatty acid accumulation in response to lipolytic agents. Applied to basal and insulin-stimulated cells, these conditions significantly decrease cell lysis and improve the yield of protein in the membrane fractions.

Determination of glucose transport activity in intact cells

The adipose cells obtained from 32 rats were incubated at 37 °C in two polypropylene containers, each of which contained a total volume of 45 ml (resulting cell concn. $1.8 \times 10^6 - 2.4 \times 10^6$ cells/ml). After incubation as indicated with 10 nM-insulin (crystalline zinc insulin, courtesy of Dr. R. E. Chance, Eli Lilly and Co.), glucose transport activity was assayed in 200 μl samples with 3-*O*-methylglucose (0.1 mM) under zero-*trans* influx conditions as previously described in detail (Karnieli *et al.*, 1981).

Preparation of plasma membranes

The remaining adipose cells were transferred into 50 ml centrifugation tubes, and spun until the centrifuge had accelerated to 1000 rev./min. The incubation buffer was withdrawn underneath the cell layer, and cells were washed with 45 ml of TES homogenization buffer (20 mM-Tris/1 mM-EDTA/255 mM-sucrose), which had been equilibrated at 18 °C. After a second spin, the washing buffer was withdrawn, and another 20 ml of TES was added. The tubes were immediately transferred to a cold-room (4 °C), and the cells were homogenized with a 50 ml Potter–Elvehjem homogenizer (A. H. Thomas Scientific, cat. no. 3431-E25; specific clearance 0.15 mm) which had been previously equilibrated to 4 °C. The temperature protocol of the cell cooling before homogenization was meticulously controlled, and the time from washing the cells until homogenization was kept at a minimum, usually less than 4 min. Because the clearance of the homogenizers varied to an extent that significantly affected the response to insulin, a standardization procedure was developed based on the time required for the pestle to fall a given distance in the homogenizer in air under the influence of gravity. This method allowed the detection of very small differences between homogenizers. Homogenizers in the middle of the range of tolerances allowed by the manufacturer were found to give the highest insulin responses. If the homogenizer clearance was too low, transport rates and number of transporters in plasma membranes from basal cells were increased, sometimes even to the values observed with plasma membranes from insulin-stimulated cells. If the homogenizer clearance was too high, then the cross-contamination of plasma membranes with low-density microsomes (microsomal fractions) increased, thereby giving rise to an increase of glucose transporter concentration without a corresponding increase in transport activity. After homogenization, plasma membranes were prepared by differential centrifugation as previously described in detail (Simpson *et al.*, 1983). For each homogenizer in use, the purity of membrane fractions was assessed by using marker enzyme activities (Simpson *et al.*, 1983).

Measurement of glucose transporter concentration

The concentration of glucose transporters in plasma membranes was assessed by using a specific D-glucose-inhibitable cytochalasin-B-binding assay as described by Wardzala *et al.* (1978). Immunoblotting of glucose transporters was performed by using an antiserum raised against the purified human erythrocyte glucose transporters (Wheeler *et al.*, 1982). Plasma-membrane proteins (40–80 μg per lane) were separated on SDS/10%-polyacrylamide gels, and proteins were transferred electrophoretically to nitrocellulose paper at 400 mA for 16 h. After blocking with albumin, the sheets were incubated with the antiserum at a dilution of 1:200 for 1 h, washed, and incubated for an additional 1 h with ^{125}I-Protein A. The sheets were extensively washed with phosphate buffer, dried, and autoradiographed for 1–3 days. Bands showing antiserum binding at 45 kDa were excised, and radioactivity was counted for exact determination of immunoreactivity. Counts were corrected for background values obtained from blank areas of the nitrocellulose sheet.

Assay of glucose transport in plasma membranes

Glucose transport in plasma-membrane vesicles was determined under equilibrium exchange conditions by a modification of a previously published method (Ludvigsen & Jarett, 1980). Samples (20–40 μg of protein) were incubated in 30 μl of TES buffer containing equal concentrations of D- and L-glucose (0.1 mM, except in kinetic studies) at 22 °C for at least 30 min, and then pulsed with an equal volume of the pulsing solution

containing approx. 10^6 c.p.m. of both L-[1-^{3}H]glucose and D-[U-^{14}C]glucose. Precise timing was achieved as follows: the pulsing solution was pipetted on the wall of a horizontally held polystyrene tube (Sarstedt, cat. no. 55-484) which contained the membrane suspension, and uptake was initiated by setting the tube vertically on to the vortex mixer. Using this procedure, the experimenter has one free hand, which can be used to add the stopping solution from a dispenser after pulse times as short as 0.5 s (measured with a metronome). Initial rates were routinely measured by stopping transport after 2.5 s with 1.0 ml of ice-cold stopping solution (0.17 mM-phloretin, 0.1 mM-D- and L-glucose in TES buffer). In the temperature-dependence studies, pulse times were varied (1–20 s) in order to obtain roughly equal filling (15–25%) of the maximum equilibration space. Stopped samples were placed on ice and filtered within 3 min on a pre-wetted Millipore 0.22 μm-pore-size membrane filter (GSWP 02500) in a 12-place vacuum manifold. To measure maximum uptake values, membranes were pulsed with a pulsing solution devoid of L-[^{3}H]glucose. After 30 min at room temperature, the samples were stopped, L-[^{3}H]-glucose was added, and the samples were filtered. Zero uptake (produced exclusively by radioactivity trapped on the filter) was measured by adding stopping solution to the membranes before adding the pulsing solution. In control experiments, the efficacy of the stopping solution was assessed: $0.43 \pm 3\%$ (means $\pm$ S.E.M. of three experiments) of the total radioactivity present within the vesicles at equilibrium was lost after 3 min, and $15.4 \pm 3\%$ after 30 min. Immediately after filtration, the filters were washed with 3×2 ml of stopping solution and put on blotting paper in order to remove as much liquid as possible. The filters were transferred to scintillation vials, and 1 ml of 0.5% Triton X-100 in water was added. The vials were shaken for 3 h, and a water-compatible scintillator was added. After correction for channel cross-over, stereospecific uptake was calculated by subtraction of L-glucose from D-glucose uptake. Initial velocities were calculated from the uptake values U_t and $U_{max.}$ as described previously (Foley *et al.*, 1978). K_m and $V_{max.}$ were calculated by linear regression of Woolf–Hofstee plots.

Calculations

All calculations were carried out on the Dartmouth Time-Sharing System computer facilities. Statistical significance was tested with a paired t test and accepted at the $P \leqslant 0.05$ level.

RESULTS

Plasma membranes prepared from maximally insulin-stimulated rat adipose cells as described in the Materials and methods section showed a markedly elevated glucose transport rate as compared with that observed in plasma membranes from basal cells. Fig. 1 illustrates time courses of stereospecific glucose uptake in plasma membranes from basal and insulin-treated cells at 22 °C. The results show that the uptake approaches equilbrium very rapidly in membranes from insulin-stimulated cells ($t_{\frac{1}{2}}$ 7 s) and that the equilibrium water spaces are identical in the membrane vesicles from both basal and insulin-stimulated cells.

In order to assess the magnitude of non-carrier-mediated sugar uptake in the vesicles, we measured

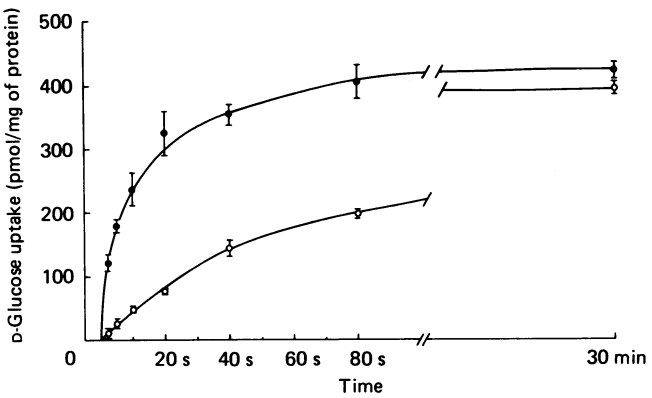

Fig. 1. Time course of stereospecific glucose uptake in plasma-membrane vesicles from basal and insulin-stimulated rat adipose cells

Isolated cells were incubated in the absence (○) or presence (●) of insulin (10 nM) as described in the Materials and methods section, and plasma membranes were prepared. Transport was assayed at 22 °C under equilibrium exchange conditions (0.1 mM-glucose). Initial velocities calculated from the curves were 5.0 (basal) and 57.0 pmol/s per mg of protein (+insulin). Data represent the means $\pm$ S.E.M. for triplicate samples in a representative experiment.

glucose uptake in the presence of phloretin (0.17 nM), which selectively inhibits stereospecific D-glucose transport. Under these conditions, initial rates of D-glucose uptake were very low (0.53 and 1.02 pmol/s per mg of protein in membrane preparations from basal and insulin-stimulated cells respectively; means of two experiments), as compared with those observed in the absence of inhibitor (5.4 and 57.0 pmol/s per mg of protein respectively). Thus the uptake of sugar by non-carrier-mediated diffusion was as low as 1.5% of the stereospecific uptake in membranes from insulin-stimulated cells and 9.6% in membranes from basal cells, indicating that the membrane vesicles were well sealed.

The substrate-dependence of D-glucose transport was studied in plasma membranes and compared with that in intact adipose cells in order to assess whether any decrease in the transport K_m contributed to the stimulatory effect of insulin. Fig. 2 shows a representative experiment from the total of four carried out. The means $\pm$ S.E.M. of the kinetic parameters were: $V_{max.}$ in cells, 7.1 ± 1.5 (basal) and 234 ± 36 fmol/min per cell (insulin); $V_{max.}$ in membranes, 1.4 ± 0.3 (basal) and 15.3 ± 4.0 nmol/s per mg of protein (insulin); K_m in cells, 8.0 ± 1.0 (basal) and 6.3 ± 1.1 mM (insulin); K_m in membranes, 17.8 ± 4.0 (basal) and 18.9 ± 2.0 mM (insulin). Thus the stimulatory effect of insulin is fully accounted for by a change in the transport $V_{max.}$ with an unchanged transport K_m.

A calculation of the effect of insulin relative to the basal value on the glucose-transport $V_{max.}$ revealed a 33-fold effect in cells, but only an 11-fold effect preserved in the plasma membranes. Surprisingly, the relative stimulatory effect of insulin in the plasma membranes was higher (15-fold), when transport in membranes was assayed at 37 °C [basal, 10.1 ± 1.4, insulin, 151 ± 40 (pmol/s per mg of protein)]. This unexpected finding suggested a different temperature-dependence of glucose

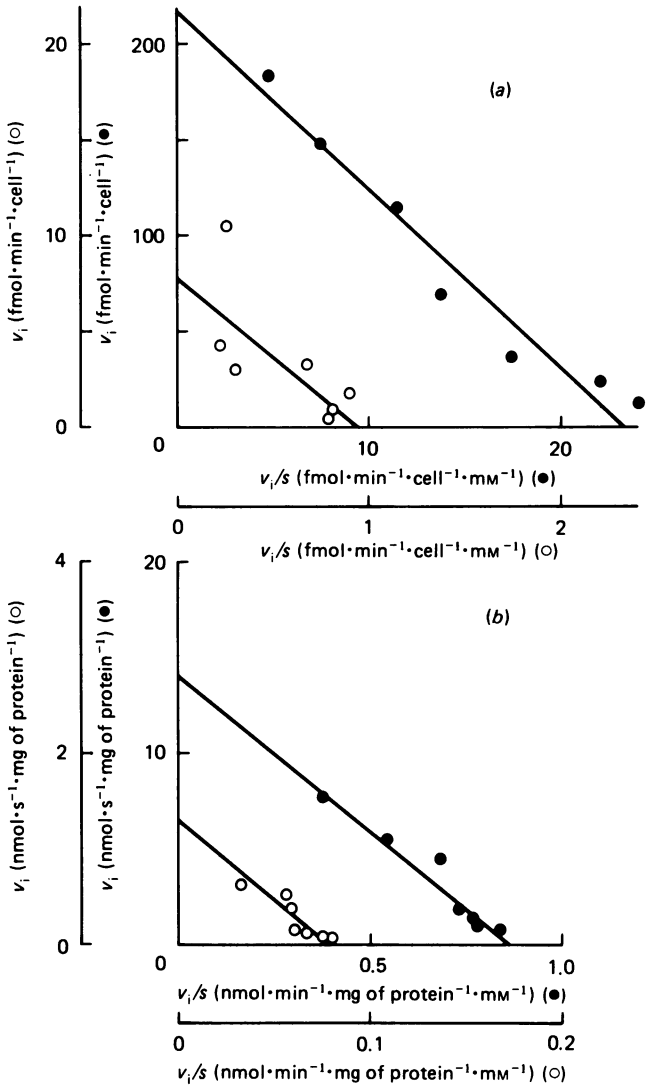

Fig. 2. Glucose-transport kinetics in intact cells (3-*O*-methylglucose) and in plasma-membrane vesicles (D-glucose) from basal and insulin-stimulated adipose cells

Isolated adipose cells were incubated in the absence (○) or presence (●) of insulin, and initial 3-*O*-methylglucose uptake rate was determined as described in the Materials and methods section (*a*). In separate experiments, plasma membranes were prepared from basal (○) and insulin-treated (●) adipose cells, and D-glucose transport was assayed as described under equilibrium exchange conditions (*b*). The Figure shows Woolf–Hofstee plots of means of triplicate samples from representative experiments which were repeated four times with different cell and membrane preparations respectively. Means ± S.E.M. of the kinetic parameters calculated from the linear regression of the plots are given in the text. Note the different scales for basal and insulin-stimulated transport.

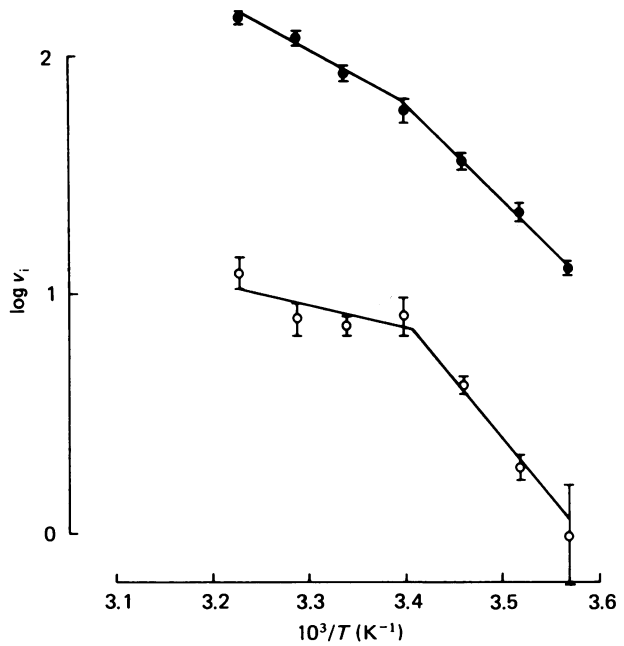

Fig. 3. Temperature-dependence of D-glucose transport in plasma-membrane vesicles from basal and insulin-stimulated rat adipose cells

Plasma membranes were prepared from basal (○) and insulin-stimulated (●) cells as described. The membranes were equilibrated with 0.1 mM-glucose at 22 °C for 30 min, and were allowed to equilibrate at the desired temperature for 5 min. Transport assays were carried out with different pulse times, which were adjusted to yield equilibration of 15–25% of the total intravesicular space within the pulse time. The data represent means ± S.E.M. for triplicate samples from a representative experiment which was repeated three times.

transport in plasma membranes from basal and insulin-stimulated cells. Fig. 3 shows the Arrhenius plot of the temperature-dependencies of plasma-membrane transport in the basal and insulin-stimulated states. Both plots exhibit a break at 21 °C. Activation energies in the low temperature range were identical in membranes from both basal and insulin-stimulated cells [86.1 ± 7.1 kJ/mol (20.5 ± 1.7 kcal/mol) and 88.6 ± 12.2 kJ/mol (21.1 ± 2.9 kcal/mol) respectively; means ± S.E.M. of three experiments]. In contrast, the activation energy over the high temperature range was significantly lower in plasma membranes from basal than from insulin-stimulated cells [27.7 ± 5.0 kJ/mol (6.6 ± 1.2 kcal/mol) and 45.3 ± 2.1 kJ/mol (10.8 ± 0.5 kcal/mol) respectively].

Table 1 shows the comparison of glucose transport activity and glucose transporter concentration in plasma membranes over a series of five separate experiments. Insulin produced a 7-fold increase in transporter concentration, as assessed by cytochalasin B binding. This increase was matched by a 9-fold stimulation of glucose transport activity in the membranes. In order to demonstrate the close correlation between cytochalasin-B-binding sites and transport activity more clearly, a separate experiment was performed in which different insulin concentrations were employed, and glucose transport activity in these plasma membranes was plotted against the corresponding concentration of transporters as described in Fig. 4. A linear regression showed a very good correlation of the two parameters.

The abscissa intercept of the curve shown in Fig. 4 represents a measure of the small portion of cytochalasin-B-binding sites which do not appear to contribute to the transport activity. This portion amounted to about 5% of the total number of cytochalasin-B-binding sites present in plasma membranes from insulin-stimulated

Table 1. Comparison of glucose transport activity and transporter concentration in membranes from basal and insulin-stimulated rat adipose cells

Isolated adipose cells were incubated in the absence or presence of insulin (10 nM), and 3-*O*-methylglucose uptake was measured at 37 °C as described in the Materials and methods section in a sample of the cell suspension. The remaining cells were homogenized, plasma membranes were prepared, and glucose transport was determined under equilibrium exchange conditions at 22 °C. A portion of the plasma membranes was used for assay of glucose-inhibitable cytochalasin B binding as described. The data represent means ± S.E.M. for five separate membrane preparations.

	Basal	+Insulin
Glucose transport in cells (fmol/min per cell)	0.06±0.01	2.4±0.2
Glucose transport in plasma membranes (pmol/s per mg of protein)	5.5±1.1	51.9±5
Concn. of cytochalasin-B-binding sites (pmol/mg)	3.6±0.5	24.8±2.5
K_D of cytochalasin B binding (nM)	83.9±12	85.0±6.5

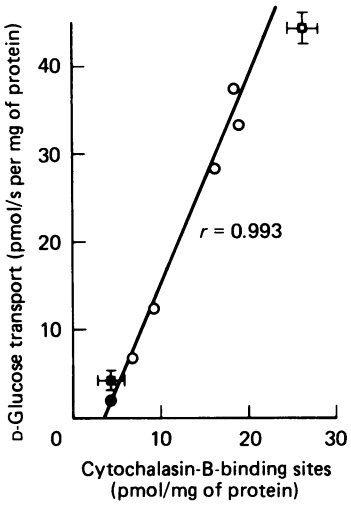

Fig. 4. Correlation between D-glucose transport activity and the concentrations of glucose transporters as assessed by cytochalasin B binding in plasma membranes from basal and insulin-stimulated rat adipose cells

Isolated adipose cells were incubated in the absence (●) or presence (○) of five different insulin concentrations. Plasma membranes were prepared, and D-glucose transport activity and cytochalasin B binding were assayed as described. Data shown as means (±S.E.M.) of values obtained in four different large-scale fractionations (basal, ■; 10 nM-insulin, □) were not included in the linear regression ($r = 0.993$).

cells. Because these binding sites might reflect the cross-contamination of plasma membranes with intracellular glucose transporters, a mixing experiment was performed in order to demonstrate that these transporters do not contribute to the plasma-membrane transport activity. Glucose transport in plasma membranes from basal cells was determined in the presence of a large excess of low-density microsomes: transport activity in the plasma membranes containing a transporter concentration of 4 pmol/mg of protein was 3.4 in the absence, and 5.8 (pmol/s per mg of protein) in the presence, of a 13-fold excess (53 pmol/mg of protein) of low-density microsomal transporters. Thus the stereospecific D-glucose uptake into intracellular membrane vesicles appears to be insignificant, and low-density microsomal glucose transporters contaminating the plasma membranes do not contribute to transport activity in the plasma-membrane fraction.

In contrast with the good correlation between glucose transport activity and transporter concentration as assessed with the cytochalasin-B-binding assay, Fig. 5 shows that the increase in transport activity was not paralleled by a comparable increase in the antibody cross-reactivity during immunoblotting. The average increase in immunoreactivity was only 2-fold, whereas the transport activity was stimulated about 10-fold in this experiment.

DISCUSSION

When glucose transport activity was assayed at 37 °C in plasma membranes from basal and insulin-stimulated rat adipose cells, a 15-fold effect of insulin was observed, compared with a 35-fold effect in the intact cells. Thus about 50% of insulin's effect would have been preserved if this difference were due to loss of the effect of insulin. However, more than 95% of insulin's effect in the intact cell would have been preserved in plasma membranes if the difference in the relative response to insulin was due to an approx. 2-fold elevation of the basal transport rate during plasma-membrane preparation. Although direct evidence is not currently available, an increase in the basal rate during homogenization appears to be the more likely reason for the quantitative difference, inasmuch as it occurred easily if homogenization temperature or homogenizer clearance was slightly varied. Further, underestimation of the basal transport rate in cells or overestimation of the rate in plasma membranes by a factor of 2 would fully explain the difference in the relative response. This possibility cannot be discounted, since the most desirable basal rates are those near the limit of sensitivity of the assay for transport in both cells and membranes. It therefore appears reasonable to conclude that a large part, if not all, of insulin's stimulatory effect in intact adipose cells can be retained in isolated plasma membranes.

During the course of our study, several variables were assessed in order to optimize the retention of the insulin response in the plasma-membrane vesicles. The crucial factors for retaining a good insulin response were homogenizer clearance and the temperature of homogenization. Homogenization temperatures lower than 17 °C increased the basal glucose transport rate in membranes, as has previously been shown to occur in intact cells through translocation of glucose transporters (Ezaki & Kono, 1982). Insufficient homogenizer clearance also gave rise to an increase in the basal transport rates. With one homogenizer, the basal glucose transport rate was as high as 50% of that in membranes from insulin-stimulated cells. The preservation of insulin's effect does

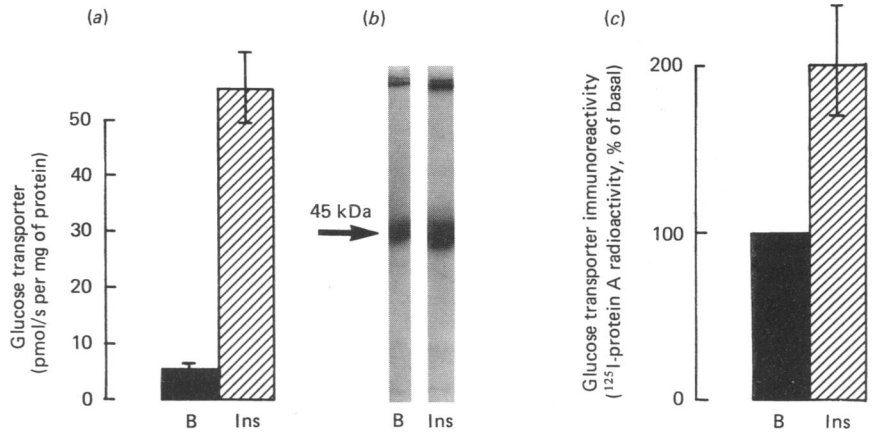

Fig. 5. Comparison of D-glucose transport activity and the concentration of glucose transporters as assessed by immunoblotting in plasma membranes from basal and insulin-stimulated rat adipose cells

Plasma membranes were prepared from basal and insulin-treated adipose cells, and D-glucose transport was determined under equilibrium exchange conditions at 22 °C (*a*). Membranes were separated on 10%-polyacrylamide gels, transferred to nitrocellulose paper, and immunoassayed with ^{125}I-Protein A as described in the Materials and methods section. (*b*) Representative autoradiograph of the immunoassay. (*c*) Quantification of the immunoblots by cutting and counting the radioactivity in the 45 kDa band. Radioactivity was normalized for basal values. The data in (*a*) and (*c*) represent means ± S.E.M. for five experiments. Key: B, basal; Ins, +insulin.

not require the presence of phosphatase inhibitors, since we failed to observe any changes in transport activity when NaF, sodium vanadate and sodium pyrophosphate were added to the homogenization buffer (results not shown). This finding is in agreement with reports by Gibbs *et al.* (1986) and our own laboratory (Joost *et al.*, 1987) showing that the glucose transporter from either basal or insulin-stimulated 3T3L1 fibroblasts or rat adipose cells respectively, does not incorporate detectable amounts of [^{32}P]phosphate. Finally, the response to insulin is also unchanged when cells are treated with KCN before homogenization; this treatment is necessary to preserve the counter-regulatory effect of catecholamines on glucose transport during homogenization of cells (Joost *et al.*, 1986).

The present data establish a very good correlation between plasma-membrane glucose transport and the number of transporters as determined with the cytochalasin-B-binding assay, when transport rates assayed at 22 °C were compared. As discussed below, the transport activation comprises two components, the second of which becomes apparent only at temperatures higher than 22 °C. At 22 °C, the effect of insulin on transport in membranes was routinely 10-fold, and that on the number of cytochalasin-B-binding sites was 7-fold. Such a difference is not surprising, since cross-contamination of plasma membranes with low-density microsomes is inevitable; this contamination would increase basal transporter concentration, but not basal activity, because the low-density microsomal transporters do not contribute to transport activity, as shown here. For example, as has previously been assessed by Simpson *et al.* (1983), consider a 5% cross-contamination of basal plasma membranes with low-density microsomes, which routinely contain 50–80 pmol of cytochalasin-B-binding sites/mg of protein. In this instance, one would have to expect an increase in the concentration of cytochalasin-B-binding sites in the plasma membranes by 2.5–4 pmol/mg of protein. This value matches the abscissa intercept in Fig. 4, which represents the concentration of sites not contributing to transport activity. Thus it appears reasonable to assume that the entire effect of insulin on glucose transport in plasma membranes assayed at 22 °C can be accounted for by the increase in cytochalasin-B-binding sites.

In marked contrast with the good correlation between plasma-membrane glucose transport and the concentration of cytochalasin-B-binding sites, the 10- and 7-fold responses of these parameters to insulin were accompanied by an only 2-fold increase in transporter concentration as assessed by immunoblotting with an antiserum against the human erythrocyte glucose transporter. This low response of transporter immunoreactivity to insulin is in agreement with all previously reported data (Wheeler *et al.*, 1982; Lienhard *et al.*, 1982; Ezaki *et al.*, 1986). Three explanations can be postulated for the striking discrepancy between the two methods to assess transporter concentration. First, the cross-reactivity of transporters with the antiserum might differ in membranes from basal and insulin-stimulated cells, possibly because of different degrees of glycosylation. In that case the cytochalasin-B-binding assay would represent the reliable method for assessment of transporter concentration. Second, a modified population of glucose transporters which do not bind cytochalasin B and do not contribute to transport activity might be present in the plasma membranes. If recognized by the antiserum, these transporters would elevate immunoreactivity in membranes from both basal and insulin-stimulated cells and thus decrease the relative response to insulin. Third, if transporters in the basal and insulin-stimulated states are assumed to cross-react identically with the antiserum, one must conclude that translocation of transporters (in that case only a 2-fold effect) accounts for only part of insulin's stimulatory effect. However, one must further conclude that cytochalasin B binding represents a functional parameter rather than a means for quantification of transporters. In other words, the activation of

transporters would create new, or expose previously blocked, cytochalasin-B-binding sites. At present no evidence is available for this rather speculative hypothesis.

Whereas the effect of insulin on glucose transport assayed in plasma membranes at 22 °C was fully accounted for by the increase in the number of transporters which bind cytochalasin B, an additional stimulatory component became apparent at 37 °C because of the different temperature-dependencies of transport in membranes from basal and insulin-stimulated cells. This finding represents a crucial piece of evidence for a qualitative difference between the transporters in the basal and insulin-stimulated states. In a previous study, Ludvigsen & Jarett (1980) reported that glucose transport in basal and insulin-stimulated plasma membranes had identical activation energies. However, the effect of insulin was very low (2–3-fold) in that study compared with that reported here, and partial activation of basal transport during preparation of plasma membranes might have concealed a difference in activation energies. It remains to be established whether this effect of insulin on the activation energy of transport, as observed here, is initiated in the low-density microsomes during the translocation process, or whether it reflects an additional mechanism of insulin action to modify the transporter in the plasma membrane.

Diverging reports have been published as to whether the stimulatory effect of insulin on glucose transport in intact adipose cells is due to a change in transport K_m or $V_{max.}$ Whereas it was generally accepted for many years that insulin changes exclusively the transport $V_{max.}$ (Taylor & Holman, 1981; Gliemann & Rees, 1983), a large K_m change has subsequently been reported (Whitesell & Abumrad, 1985), which depended on strict avoidance of any agitation of the cells. More recently, two additional studies were published which re-examined the kinetic parameters, and again conflicting data were obtained (Martz *et al.*, 1986; Toyoda *et al.*, 1987). It has thus become obvious that the incubation conditions, mainly the pattern of cell agitation and the cell concentration, may determine whether the effect of insulin on transport K_m is observed. In the present study, the cells were moderately shaken (40 cycles/min) and a cell concentration of approx. 2×10^6/ml was not exceeded, because higher cell concentration and incubation without any agitation were thought possibly to give rise to accumulation of metabolites which might modify the transort activity. Under these conditions, no change in the transport K_m was detected either in cells or in plasma membranes prepared from these cells. These results clearly demonstrate that a large, at least 10-fold, effect of insulin is accounted for exclusively by a change in the transport $V_{max.}$

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REFERENCES

Carter-Su, C. & Czech, M. P. (1980) J. Biol. Chem. **255**, 10382–10386
Cushman, S. W. & Wardzala, L. J. (1980) J. Biol. Chem. **255**, 4758–4762
Ezaki, O. & Kono, T. (1982) J. Biol. Chem. **257**, 14306–14310
Ezaki, O., Kasuga, M., Akanuma, Y., Takata, K., Hirano, H., Fujita-Yamaguchi, Y. & Kasahara, M. (1986) J. Biol. Chem. **261**, 3295–3305
Foley, J. E., Cushman, S. W. & Salans, L. B. (1978) Am. J. Physiol. **234**, E112–E119
Gibbs, E. M., Allard, W. J. & Lienhard, G. E. (1986) J. Biol. Chem. **261**, 16599–16603
Gliemann, J. & Rees, W. D. (1983) Curr. Top. Membr. Transp. **18**, 339–379
Honnor, R. C., Dhillon, G. S. & Londos, C. (1985) J. Biol. Chem. **260**, 15122–15129
Joost, H. G., Weber, T. M., Cushman, S. W. & Simpson, I. A. (1986) J. Biol. Chem. **261**, 10033–10036
Joost, H. G., Weber, T. M., Cushman, S. W. & Simpson, I. A. (1987) J. Biol. Chem. **262**, 11261–11267
Karnieli, E., Zarnowski, M. J., Hissin, P. J., Simpson, I. A., Salans, L. B. & Cushman, S. W. (1981) J. Biol. Chem. **256**, 4772–4777
Lienhard, G. E., Kin, H. K., Ransome, K. J. & Gorga, J. C. (1982) Biochem. Biophys. Res. Commun. **105**, 1150–1156
Ludvigsen, C. & Jarett, L. (1980) Diabetes **29**, 373–378
Martin, D. B. & Carter, J. R. (1970) Science **167**, 874–877
Martz, A., Mookerjee, B. K. & Jung, C. Y. (1986) J. Biol. Chem. **261**, 13606–13609
Rodbell, M. (1964) J. Biol. Chem. **239**, 375–380
Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B. & Cushman, S. W. (1983) Biochim. Biophys. Acta **763**, 393–407
Suzuki, K. & Kono, T. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2542–2545
Taylor, L. P. & Holman, G. D. (1981) Biochim. Biophys. Acta **642**, 325–335
Toyoda, N., Flanagan, J. E. & Kono, T. (1987) J. Biol. Chem. **262**, 2737–2745
Wardzala, L. J., Cushman, S. W. & Salans, L. B. (1978) J. Biol. Chem. **253**, 8002–8005
Wheeler, T. J., Simpson, I. A., Sogin, D. C., Hinkle, P. C. & Cushman, S. W. (1982) Biochem. Biophys. Res. Commun. **105**, 89–95
Whitesell, R. R. & Abumrad, N. A. (1985) J. Biol. Chem. **260**, 2894–2899
Whitesell, R. R. & Abumrad, N. A. (1986) J. Biol. Chem. **261**, 15090–15096

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