Insulin stimulation of glucose transport in isolated rat adipocytes

Functional evidence for insulin activation of intrinsic transporter activity within the plasma membrane

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We examined the effects of the membrane-impermeant amino-group-modifying agent fluorescein isothiocyanate (FITC) on the basal and insulin-stimulated hexose-transport activity of isolated rat adipocytes. Pre-treatment of cells with FITC causes irreversible inhibition of transport measured in subsequently washed cells. Transport activity was inhibited by approx. 50% with 2 mM-FITC in 8 min. The cells respond to insulin, after FITC treatment and removal, and the fold increase in transport above the basal value caused by maximal concentrations of insulin was independent of the concentration of FITC used for pre-treatment over the range 0–2 mM, where basal activity was progressively inhibited. The ability of FITC to modify selectively hexose transporters accessible only to the external milieu was evaluated by two methods. (1) Free intracellular FITC, and the distribution of FITC bound to cellular components, were assessed after dialysis of the homogenate and subcellular fractionation on sucrose gradients by direct spectroscopic measurement of fluorescein. Most (98%) of the FITC was associated with the non-diffusible fractions. Equilibrium sucrose-density-gradient centrifugation of the homogenate demonstrated that the subcellular distribution of the bound FITC correlated with the density distribution of a plasma-membrane marker, but not markers for Golgi, endoplasmic reticulum, mitochondria or protein. Exposing the cellular homogenate, rather than the intact cell preparation, to 2 mM-FITC resulted in a 4–5-fold increase in total bound FITC, and the density-distribution profile more closely resembled the distribution of total protein. (2) Incubation of hexokinase preparations with FITC rapidly and irreversibly inactivates this protein. However, both intracellular hexokinase total activity and its apparent Michaelis constant for glucose were unaffected in FITC-treated intact cells. Further control experiments demonstrated that FITC pre-treatment of cells had no effect on the intracellular ATP concentration or the dose–response curve of insulin stimulation of hexose transport. Since the fold increase of hexose transport induced by insulin is constant over the range of inhibition of surface-labelled hexose transporters, we suggest that insulin-induced insertion of additional transporters into the plasma membrane may not be the major locus of acceleration of hexose transport by the hormone.

INTRODUCTION

In spite of extensive studies, the mechanism of insulin action at the molecular level is an unresolved problem (Levine, 1982; Czech, 1980; Hilf *et al.*, 1981; Fain, 1984; Horn & Walaas, 1984). The binding of insulin to surface-membrane receptors leads to alterations in the activities of numerous membrane-associated functions, such as the transport of sugar, amino acids and various ions, and the activities of numerous membrane enzymes. The insulin receptor is phosphorylated, after interaction with hormone, and also expresses tyrosyl kinase activity (Kahn *et al.*, 1985; Pilch *et al.*, 1985). Recent studies also indicate that insulin is capable of directly altering gross structural properties of surface membranes. The hormone causes membrane ruffling and reorganization of actin in KB cells (Goshima *et al.*, 1984), induces surface projections in fibroblasts (Sonenberg, 1977), and is capable of influencing the membrane lipid structure of certain bilayer domains (Luly & Shinitzky, 1979; Hyslop *et al.*, 1984b). Multiple effects of insulin have also been

found on the phosphorylation of plasma-membrane proteins (Horn & Walaas, 1984; Denton *et al.*, 1981; Houslay, 1981; Houslay & Heyworth, 1983).

For the sugar-transport system in insulin-sensitive cells, the stimulation process by hormone requires the presence of an intact cell and is time- (Ciaraldi & Olefsky, 1983), oxygen- (Walaas & Walaas, 1952) and ATP-dependent (Kono *et al.*, 1977; Chandramouli *et al.*, 1977). These observations support the view that the hormone activates sugar uptake not simply via direct interaction with the membrane transport system but by a more complex indirect mechanism.

Data from several laboratories studying the properties of plasma- and subcellular-membrane preparations suggest that treatment with insulin gives rise to a decrease of D-glucose-displaceable cytochalasin B-binding sites within the endoplasmic reticulum/Golgi fraction, with a concomitant increase in these sites within the plasma membrane of adipocytes (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Karnieli *et al.*, 1981; Ezaki & Kono, 1982; Simpson *et al.*, 1983; Oka & Czech, 1984).

Abbreviations used: FITC, fluorescein isothiocyanate; ¹²⁵I-WGA, ¹²⁵I-labelled wheat-germ agglutinin.

and in placental membranes (Wardzala & Jeanrenaud, 1983). These data have been largely interpreted as the hormone causing translocation of hexose transporters from an internal pool to their functionally active conformation in the plasma membrane, thereby accelerating net cellular uptake of hexose. Interestingly, in another study, activation of hexose transport in fat-cells has been suggested to be due to removal of an inhibitory protein from the plasma membrane (Schoenle *et al.*, 1984).

Functional data in support of the translocation model are, however, at present insufficient to demonstrate rigorously a rapid insulin-dependent migration of transporter protein. Indeed, Carter-Su & Czech (1980) reported that insulin stimulates the total activity of transporters in a combined plasma-membrane/endoplasmic-reticulum fraction. Thus it seemed that insulin might activate endogenous transporters independently of redistribution from one membrane to another (Fain, 1984). The present study was designed to provide functional evidence to detect any up-regulation of hexose transporters into the plasma membrane by insulin activation of adipocytes.

Isothiocyanates have been utilized in a number of studies to inhibit hexose transport (Weber & Semenza, 1983; Peerce & Wright, 1984). FITC has been utilized to modify covalently cell-surface proteins in fibroblasts (Edidin *et al.*, 1976) and erythrocytes (Peters *et al.*, 1974). In both of these studies, labelling of cellular proteins was confined primarily to the plasma membrane. Measurement of the magnitude of the fold stimulation of hexose transport by insulin, over a wide range of inhibited transport rates in adipocytes washed free of FITC, could therefore be used to monitor the appearance of any uninhibited transporters at the cell surface, translocated from an intracellular site in response to insulin provided that the agent does not interfere with the insulin stimulation process. Our data provide functional evidence that insulin accelerates the maximal velocity of glucose uptake into rat adipocytes either by direct activation of basal transporters or by activating nascent transporters already located at the cell surface.

MODELS OF INSULIN STIMULATION OF GLUCOSE TRANSPORT

It is generally held that the facilitated transport of glucose through the cell surface membrane follows Michaelis-Menten kinetics (Morgan & Neely, 1972) and may be represented by a substrate-carrier-complex model, thus:

where S_0 is the concentration of glucose outside the cell, C is the concentration of glucose transporters in the membrane, SC is the concentration of activated complex, and S_i is the concentration of glucose transported inside the cell.

Since typically $C \ll S_0$ and $k_{+2} < k_{+1}$, the initial rate of transport reflects the relative numbers of functioning transport proteins in the membrane, thus:

$$V_B = \frac{C_B \cdot k_{+2}}{1 + \frac{K_m}{S_0}} \quad (2)$$

where V_B is the basal rate of transport, C_B represents the concentration of transporters in the membrane exhibiting basal (unstimulated) activity, and K_m is the Michaelis constant.

$$K_m = \frac{S_0 \cdot C}{SC} = \frac{k_{+1} + k_{+2}}{k_{-1}} \quad (3)$$

Insulin has been proposed to increase maximal transport capacity in the adipocyte (and other cell types) either by increasing the number of activated transport units (Model A), or by increasing the inherent activity of already functioning units in the membrane (Model B), thereby changing the permeation constant (k_2') associated with the transporter.

Model A: increased transporter number

If insulin increases the number of activated transport units without affecting K_m , then, for Model A, the sum of basal and insulin-stimulated rates may be represented as follows:

$$V_I = V_B + \frac{C_I \cdot k_2'}{1 + \frac{K_m}{S_0}} = \frac{C_B \cdot k_2}{1 + \frac{K_m}{S_0}} + \frac{C_I \cdot k_2'}{1 + \frac{K_m}{S_0}} \quad (4)$$

where V_I is the transport rate observed in the cell after stimulation of transport activity by insulin, C_I is the concentration of transporters functioning in the membrane because of the influence of insulin. It is generally held for this model that newly activated or inserted transporters are functionally identical (Cushman & Wardzala, 1980), and in this case $k_2 = k_2'$ and eqn. (4) becomes:

$$V_I = \frac{(C_B + C_I)k_2}{1 + \frac{K_m}{S_0}} \quad (5)$$

[Note that, in the absence of insulin, $C_I = 0$ and eqn. (4) reduces to $V_I = V_B$.]

Model B: direct activation of membrane transport

The insulin-stimulated rate may be expressed differently for Model B, where the inherent activity of transporters is influenced more directly by insulin (I). Although the mechanism by which insulin stimulates transport is unknown, the overall effects on the transport system exhibit characteristics that may be compared with regulation of a V-series allosteric enzyme (Martin *et al.*, 1981), where insulin binding to the membrane leads to an increase in transport maximal velocity without affecting transport K_m (Olefsky, 1978). Here,

It is generally assumed here that all basal transporters may be converted into activated units by saturating insulin concentrations (i.e. $C_B = 0$ at maximal stimulation, and C_B in the absence of insulin equals C_I in the presence of maximally stimulating insulin). Thus, at saturating insulin:

$$V_I = \frac{C_I \cdot k_2'}{1 + \frac{K_m}{S_0}} \quad (7)$$

Model assessment criteria

Aspects of the above models could be evaluated with agents that irreversibly inhibit plasma-membrane surface carriers without affecting intracellular carriers or the process by which insulin activates hexose transport. If V_B and V_B' and V_I and V_I' are the basal and insulin-stimulated transport rates in the absence and presence of inhibitory agent, then the fractional inhibited basal transport (i) is V_B'/V_B and the fold stimulation above basal by saturating insulin is $V_I/V_B = \sigma$ in the absence, and $\sigma' = V_I'/V_B'$ in the presence, of inhibitor respectively.

If insulin caused transporters to be inserted into the plasma membrane from an internal environment protected from the agent (Model AI) (or alternatively caused activation of new transporters already in the plasma membrane that were insensitive to inhibition by the agent), dividing eqns. (2) and (5), then, in the absence of inhibition:

$$\sigma_{[AI]} = \frac{C_B + C_I}{C_B} \quad (8)$$

Repeating the above analysis for cells pretreated with surface transport inhibitor, the fold stimulation is:

$$\sigma'_{[AI]} = \frac{(iC_B + C_I)}{iC_B} = 1 + \frac{C_I}{C_B} \cdot i^{-1} \quad (9)$$

Thus the fold insulin stimulation will be a hyperbolic function of the fractional inhibited basal transport rate (Model AI).

If, however, insulin were to stimulate transporters (inactive in the basal state) within the plasma-membrane matrix that are inhibited to the same degree as basal transporters by preincubation with inhibitor, then:

$$\sigma'_{[AII]} = \frac{iC_B + iC_I}{iC_B} = 1 + \frac{C_I}{C_B} \quad (10)$$

Thus the magnitude of insulin stimulation of hexose transport will be independent of the fractional inhibited transport rate.

Finally, for Model B, where insulin directly activates existing basal transporters within the plasma membrane, dividing eqn. (7) by eqn. (2):

$$\sigma'_{[B]} = \frac{iC_I k_2'}{iC_B k_2} = \frac{k_2'}{k_2} \quad (11)$$

Again, the magnitude of insulin stimulation of transport will be independent of the fractional inhibited transport rate.

The above discussion indicates that, for any agent that fulfils the aforementioned criteria, basal and insulin-stimulated hexose-uptake data after exposure to the agent may distinguish Model AI from Models AII and B.

MATERIALS AND METHODS

Preparation of adipocytes

Adipocytes were isolated from male Sprague-Dawley rats (all approx. 200 g wt.) (Simonsen Laboratories, Gilroy, CA, U.S.A.) by digesting minced epididymal adipose tissue in buffer containing NaCl (110 mM), KCl (5 mM), CaCl₂ (1 mM), MgSO₄ (2.5 mM), KH₂PO₄ (1 mM), Mops (25 mM) (Sigma, St. Louis, MO, U.S.A.), 20 mM-NaOH, pH 7.4 ('physiologic Mops'), with 4% (w/v) albumin (fatty-acid-free; Sigma) and 1 mg of

collagenase (lot CLS 43N131; Worthington, Freehold, NJ, U.S.A.)/ml (Hyslop *et al.*, 1984a; Sauerheber *et al.*, 1984a). Cells were filtered and washed in Mops buffer containing 1% albumin (approx. 305 mosm) and then diluted to approx. 10⁶ cells/ml of buffer. Cell counts were determined by direct microscopic examination of small samples of cell suspension diluted 10-fold.

2-Deoxy-D-glucose uptake measurements

The initial rate of the uptake of 2-deoxy-D-[³H]glucose was measured essentially as described by Gliemann *et al.* (1972) and Olefsky (1978). Cells were incubated at a fixed temperature (37 ± 0.2 °C) either with or without insulin (40 ng/ml) (Sigma) for 10 min before addition of labelled substrate (0.1 mM; final sp. radioactivity 4 Ci/mol unless indicated otherwise). Uptake was terminated after 10 min by centrifuging 200 µl portions of cell suspension through dinonyl phthalate oil in 400 µl centrifuge tubes in an Eppendorf Microfuge (Brinkmann) for 3 s. The tubes were sliced at the cell/oil interface, and counted for ³H radioactivity in Aquasol (New England Nuclear, Boston, MA, U.S.A.) in a Searle liquid-scintillation counter. 2-Deoxy-D-glucose passes through the membrane and is phosphorylated by the same mechanisms as for D-glucose, but is not further metabolized inside the cell (Wick *et al.*, 1957). Uptake of this glucose analogue under the conditions used directly measures the membrane transport process in both the absence and the presence of insulin (Sauerheber *et al.*, 1984b). Trapped and non-carrier-mediated uptake of 2-deoxy-D-glucose was estimated by the inclusion of 50 µM-cytochalasin B in the assay media.

Fluorescein isothiocyanate effects on transport

FITC modification of amino groups on proteins and amino phospholipids (Thelen *et al.*, 1984) is thought to occur by the formation of acylalkyl-thiourea or acylamide conjugates (Cary & Sundberg, 1983). pH 9 buffers are generally employed for amino-group modification by FITC, although the reaction proceeds at neutral pH as well (Pabst *et al.*, 1983). However, at low pH thiol groups may also react with FITC, as noted for phenyl isocyanate (Fraenkel-Conrat, 1944). Agents that modify thiol groups are known to inhibit the process by which insulin activates hexose transport (May, 1985). In the present study we demonstrate that FITC can be used as a poorly permeant covalent inhibitor of glucose transport in adipocytes that does not appear to interfere, at pH 9.2, with the process by which insulin activates hexose transport.

Cells were washed into 'physiologic Mops' containing 5 mM-sodium borate at pH 9.2, and were divided into equal portions (determined by counting cells, as above). Control cells received ethanol carrier, and experimental tubes were treated with fluorescein isothiocyanate (type II; Sigma; dissolved in ethanol) so that the final concentration was 0–2 mM (ethanol was < 250 mM). Control and experimental cells were incubated at 37 °C for 8 min and were diluted with 'physiologic Mops' buffer, pH 7.4, and washed in parallel (until all yellowish colour was absent from experimental cells). Photomicrographs (magnification × 40) of adipocytes taken after exposure to FITC under these conditions indicated no detectable cell breakage above the extent noted in the control (e.g. ethanol-carrier-treated) cells (results not shown) (see also Sauerheber *et al.*, 1984b). Hexose uptake was then measured in the pre-treated washed, control and

experimental cells as described above, in quadruplicate tubes. Some of the high-range inhibition data were obtained by diluting cells in 'physiologic Mops' buffer (after stopping hexose uptake with 1 mM-HgCl₂ and before centrifuging through oil) to decrease the extracellular trapped label amount to a suitably low (20%) fraction of the basal counts observed.

Hexose uptake under the above conditions, both with and without FITC incubation in the presence and absence of insulin, was a linear function of time up to 20 min incubation at 37 °C, and its plot extrapolated through the origin. Uptake, under these conditions, in the presence of maximally stimulating insulin concentrations (40 ng/ml), represents the maximum activation achievable by hormone in either the presence or the absence of FITC pre-treatment. Control experiments (results not shown) indicated that FITC treatment had no effect on the efficacy of cytochalasin B to inhibit transport.

Preparation of adipocyte homogenates for subcellular fractionation

Adipocytes (2×10^6 – 5×10^6) were incubated with 20–50 nCi of ¹²⁵I-WGA for 5 min at 37 °C in a total volume of 7 ml. [WGA was from EY Laboratories, San Mateo, CA, U.S.A., and radioiodinated as described by Jesaitis *et al.* (1983).] The cell suspension was collected by flotation, and homogenized in 20 ml of ice-cold 250 mM-sucrose/1 mM-EGTA/100 μM-MgCl₂/10 mM-Mops, pH 7.4 (sucrose buffer). The homogenate was transferred to a Sorvall Ti50 rotor (4 °C), and centrifuged at 100 000 *g* for 35 min. The pellet was re-homogenized in 3–5 ml of sucrose buffer before isopycnic density-gradient centrifugation. For studies involving treatment of the homogenate with FITC, the pellet was resuspended in 14 ml of 25 mM-sodium borate (pH 9.2)/250 mM-sucrose/100 μM-MgCl₂/1 mM-EGTA. To a 7 ml portion was added FITC in ethanol to a final concentration of 2 mM, or ethanol carrier alone to the other sample, and the homogenates were incubated for 8 min at 37 °C. At the end of this period, 0.7 ml of 100 mM-Mops/250 mM-sucrose/100 μM-MgCl₂ was added to each tube, and the pH of the mixture was rapidly adjusted to 7.0 with 0.1 M-HCl. Then 20–50 nCi of ¹²⁵I-WGA was added, and the homogenate further incubated for 5 min at 37 °C. The homogenate was then cooled on ice to 3 °C, diluted to 40 ml with sucrose buffer at 3 °C, and centrifuged at 100 000 *g* for 35 min. The pellet was washed once in approx. 50 ml of buffer and re-sedimented and then resuspended in 3–5 ml of buffer before isopycnic density-gradient centrifugation.

Subcellular fractionation by isopycnic sedimentation in sucrose density gradients

Linear sucrose gradients were prepared 12 h before use in Dupont 36 ml polyallomer centrifuge tubes. The gradient [26 ml of 55–15% (w/w) sucrose] was layered over a 3 ml cushion of 60% (w/w) sucrose, all in 100 μM-MgCl₂/1 mM-EGTA/10 mM-Mops, pH 7.4, as the diluent. Homogenate (5 ml) was layered on top of the gradient. Gradients were centrifuged for 70 min in a Sorvall TV 850 vertical rotor at 45 000 rev./min. Gradients were pumped out with Fluorinert FC 40 (Sigma) into 0.75 ml fractions. The sucrose concentration in each fraction was determined by measurement of the refractive index of a 30 μl sample with a Zeiss refractometer.

Measurement of subcellular markers in the gradient fractions

Fractions were analysed as follows. (A) FITC was quantified by measurement of fluorescein fluorescence in an SLM 8000 spectrophotometer. Excitation wavelength was 490 nm, with 4 nm bandpass. Emission intensities were observed through a Corion 520 three-cavity interference filter. A 25 μl portion of each fraction was added to 1.5 ml of 10 mM-Mops, pH 7.4, in a stirred cuvette. Fluorescein fluorescence was quantified as the acid-quenchable intensity (90–95% of total) compared with fluorescein standard (10 μM). (B) ¹²⁵I-WGA was measured in each fraction by counting radioactivity of each fraction in a Searle γ-radiation counter as a marker for plasma membrane (Cuatrecasas, 1973). (C) NADPH:cytochrome *c* reductase (cyanide-insensitive) was measured by the method of Philips & Langdon (1962) as a marker for endoplasmic reticulum. The rate of cytochrome *c* reduction was measured by diluting 100 μl fractions into the assay medium and monitoring changes in the *A*₅₅₀. (D) Adenylate kinase activity (a marker for intact mitochondria) was measured as described by Melnick *et al.* (1979). (E) UDP-galactosyltransferase (a marker for Golgi) was assayed as described by Baxter & Durham (1979), except that (i) ovomucoid was substituted as the acceptor protein, and (ii) after termination of the assay, radiolabelled galactosylated ovomucoid in each gradient sample was purified from [¹⁴C]UDP-galactose by equilibrium dialysis against 4 × 50 vol. of 1 mM-EGTA/10 mM-Mops, pH 7.4. (F) Protein was determined by the micro-titre Bradford (1976) procedure.

Measurement of intracellular ATP in adipocytes

Adipocytes (2×10^4) were withdrawn in 100 μl samples and added to 2.0 ml of 10 mM-KH₂PO₄/4 mM-MgSO₄, pH 7.4, at 95–100 °C. After 4 min, samples were frozen and assayed the next day for ATP by the method of Stanley & Williams (1969), by using luciferase/luciferin from Calbiochem. Photon counting was performed on a Beckman LS8000 liquid-scintillation spectrometer in its out-of-coincidence mode. ATP was standardized with ATP solutions treated in parallel with samples. ATP was from Sigma.

Hexokinase activity measurements in adipocytes

FITC-treated or control adipocyte suspensions (3×10^6 /ml) were homogenized in 10 mM-Tris (pH 8.0)/1 mM-EGTA/100 μM-MgCl₂ at 4 °C and centrifuged at 10 000 *g* for 10 min. Portions (50 μl) were added to 1 ml of the above-mentioned assay buffer. A 10 μl portion of assay mixture was withdrawn and rapidly mixed with 200 μl of ethanol/1 mM-Na₂HPO₄ (pH 7.0) (7:3, v/v) at 4 °C. Hexokinase activity was assayed essentially as described by Beutler (1971), except that NADPH produced by the coupled assay to glucose-6-phosphate dehydrogenase was measured by the NADPH-dependent luciferin/luciferase assay with the FMN/decylaldehyde cofactors available from Sigma. A 10 μl portion of the ethanolic solution was added to the luciferase 'cocktail', and photon counting was performed as described for ATP assay. A time course of NADPH generation was performed over 30 min, and was made quantitative with NADPH standard solutions treated in a similar manner.

Direct effects of FITC on hexokinase activity in adipocyte homogenates and purified yeast enzyme

Yeast hexokinase (Sigma) was assayed in the coupled assay system exactly as described by Beutler (1971). The enzyme was incubated in 10 mM-sodium borate, pH 9.2, at 100 munits/ml in the presence and absence of 1 mM-FITC. At various time intervals, a 100 μ l sample was taken for spectrophotometric assay of hexokinase activity (rate of NADPH generation measured at 340 nm). Adipocyte homogenates were prepared as described above, and diluted 1:5 in sodium borate buffer. The pH of the mixture was adjusted to pH 9.2 with 1 M-NaOH, and FITC was added in ethanol to give final concentrations of 0, 0.5, 1.0 and 2.0 mM. After 2 min, 100 μ l of this mixture was added to hexokinase assay buffer as described in the preceding section.

RESULTS

Fluorescein isothiocyanate inhibition of hexose transport

FITC preincubation with adipocytes demonstrated a dose-dependent inhibition of hexose transport. The magnitude of inhibition at any fixed dose varied from experiment to experiment, but, in general, incubation with 2 mM-FITC produced 50% inhibition after about 8 min exposure to adipocytes. After FITC pre-treatment and removal, both basal and insulin-stimulated uptake rates of 2-deoxy-D-glucose were linear as a function of time. Fig. 1 shows the data obtained from four separate experiments, performed after FITC treatment of the cells. The percentage inhibition of basal transport was assessed by comparison with uptake rate in cells treated in parallel to FITC-treated cells, pre-exposed to pH 9.2 buffer with ethanol carrier. The fold stimulation above the basal rate is plotted as a function of the fractional inhibited basal transport rate. Fig. 1 also shows theoretical curves obtained by substituting values into eqns. (8), (9) and (10). σ has been consistently observed to have a mean value of 2 (± 0.35) for insulin-stimulated 2-deoxyglucose uptake into rat adipocytes under our experimental conditions. We have previously demonstrated that these values are comparable with those obtained for insulin-stimulated overall uptake of glucose from the media by these cells over long time courses (Sauerheber *et al.*, 1984b). Moreover, the stimulation by insulin of label uptake is identical whether labelled deoxyglucose is mixed with unlabelled 2-deoxy-D-glucose or with unlabelled D-glucose, so that the added hexose substrate is 100% 2-deoxyglucose or 99.98% D-glucose, respectively. This suggests that any accumulation of free 2-deoxyglucose does not influence the activity of the hexose uptake rate-determining (i.e. transport) step.

Intracellular ATP and hexokinase measurements after FITC treatment of adipocytes

The effects of FITC treatment of intact adipocytes are shown in Tables 1 and 2. No apparent changes were observed in either total intracellular ATP or the maximal rates of glucose phosphorylation by the homogenates. Furthermore, the apparent Michaelis constant of hexokinase phosphorylation of glucose was not altered within limits of experimental error (Fig. 2). In contrast, when adipocyte homogenates were treated with FITC, and assayed for hexokinase activity, glucose phosphorylation was progressively inhibited as a function of FITC

Fig. 1. Stimulation of 2-deoxy-D-glucose uptake by insulin ($\bullet$), ± 1 S.D., as a function of the fractional inhibited basal transport (i) by preincubation of adipocytes with FITC

The continuous lines present curve-fit data for eqn. (9) (Model A_I), eqn. (10) (Model A_{II}), and eqn. (11) (Model B). A_I represents activation of transport by a mechanism involving insertion of transporters from inside the cell into the surface membrane. A_{II} represents activation of membrane transporters that are not functional in the basal state, and B represents a direct activation of inherent activity of basal transporters within the surface membrane (see the text). The broken line represents the mean value (± 1 S.D.) of the data points ($= 1.9 \pm 0.2$). The mean value of control insulin stimulation of transport (± 1 S.D.) (in the absence of FITC), averaged from measurements made in parallel with each data point above, was 2.0 ± 0.35 ($n = 14$), and is indicated at $i = 1$. See the text for details of cell isolation, drug-exposure conditions, and hexose-uptake measurement procedures.

concentration (Table 3). Hexokinase inhibition by all doses of FITC in adipocytes was maximally achieved after 2 min exposure. Similar observations were obtained with the highly purified enzyme from yeast (Fig. 3), supporting the concept that FITC-conjugated hexokinase shows decreased activity.

Table 1. Intracellular ATP content of FITC-modified adipocytes

Intracellular ATP concentrations in adipocytes were measured as indicated in the Materials and methods section after exposure of cells to 1 mM- or 2 mM-FITC for 10 or 20 min.

[FITC] (mM)	Exposure time (min)	ATP content (nmol/10 ⁶ cells)
0 (control)	10	15.4
	20	14.1
1	10	13.8
	20	14.3
2	10	15.3
	20	13.1

Table 2. Marker-enzyme activities, total protein and FITC bound to adipocytes after treatment of either intact cells or homogenates with 2 mM-FITC for 8 min

Intact adipocytes and homogenates were prepared and FITC-treated, and all listed parameters were measured, as indicated in the Materials and methods section. Data are from one representative experiment of a total of three.

Marker	Quantity	Major peak sedimentation densities (g/ml)
NADPH:cytochrome <i>c</i> reductase (nmol/min per 10 ⁶ cells)	Control 6.5 FITC-treated 6.8	1.0810, 1.1663 —
UDP-galactosyltransferase (nmol/min per 10 ⁶ cells)	FITC-treated 3×10^{-7}	1.2238, 1.2025
Adenylate kinase (nmol/min per 10 ⁶ cells)	Control 56.2 FITC-treated 67.3	— 1.1713
¹²⁵ I-WGA	Cells	1.1366
	Homogenate	1.1318
FITC bound (nmol/10 ⁶ cells)	Cells 0.76 Homogenate 3.42	1.1400 1.0700, 1.222, 1.1980
Protein (mg/10 ⁶ cells)	Control 2.1 FITC-treated 2.2	1.1764, 1.1190

Fig. 2. Hexokinase activity of adipocyte homogenates as a function of glucose concentration

Homogenates of control and FITC-pretreated adipocytes were prepared as indicated in the Materials and methods section. Activity is expressed as nmol of NADPH produced in a coupled assay/min per 10⁶ cells: ○, cells pretreated with 2 mM-FITC for 8 min; ●, control cells incubated with ethanol carrier.

Table 3. Effects of FITC on hexokinase activity in adipocyte homogenates

Adipocyte homogenates were prepared from cells exposed to various concentrations of FITC for 2 min at pH 9.2 (see the text). Hexokinase activity was assessed by diluting the modification medium into the assay buffer, as described in the Materials and methods section.

[FITC] (mM)	Hexokinase activity (% of control)
0 (control)	100
0.5	75
1.0	58
2.0	29

Fig. 3. Inhibition of purified yeast hexokinase by incubation with either ethanol carrier (●) or 1 mM-FITC (○) at pH 9.2

Hexokinase activity was measured with or without FITC as indicated in the text.

Fig. 4. Dose response of 2-deoxyglucose uptake to insulin in adipocytes (10⁵ cells) preincubated at pH 9.2 with either 2 mM-FITC for 8 min (○) or ethanol carrier (●)

Cells were prepared, FITC-treated, and washed free of FITC before the hexose-transport assay.

Dose-response effects of insulin on activation of hexose transport in FITC-treated adipocytes

The data presented in Fig. 4 demonstrate that, although FITC treatment inhibits the basal and insulin-stimulated transport of 2-deoxy-D-glucose, (in this case, approx. 56%) the sensitivity of the activation mechanism to insulin is not altered. Identical findings were observed for a separate experiment (not shown) at 50% inhibition by FITC. The dose-response curves were superimposable with control curves.

Subcellular fractionation by isopycnic sedimentation in sucrose density gradients

Control or FITC-treated (2 mM, 8 min) adipocytes were homogenized as outlined in the Materials and methods section and sedimented on sucrose gradients. After fractionation of the 30 ml gradient into 40 0.75 ml fractions, enzyme markers, FITC fluorescence and the mean sucrose concentration in each fraction were determined as outlined in the Materials and methods section, and plotted as the percentage of the total in each fraction as a function of the sucrose concentration (Fig. 5). We demonstrated that the labelling pattern with cell-surface marker ^{125}I -WGA was identical whether the ^{125}I was bound to intact cell surfaces or added after homogenization, confirming the use of this lectin as a plasma-membrane marker in these cells (results not shown). The sedimentation profile of the plasma membrane is unique among the organelles measured in this study (endoplasmic reticulum, Golgi, mitochondria).

Measurement of fluorescein fluorescence in the gradient fractions from cells pretreated with FITC gave sedimentation profiles almost identical with those obtained for ^{125}I -WGA on either controls or cells pretreated with FITC. The profiles given in Fig. 5 are obtained from cells treated with FITC for 8 min. Identical distributions of ^{125}I -WGA and FITC were obtained for cells treated for 15 min (results not shown). The maximal activities of the enzymes, ^{125}I -WGA binding and fluorescein-fluorescence peaks are given in Table 2. When homogenates were treated with 2 mM-FITC for 8 min and then sedimented on sucrose gradients, the ^{125}I -WGA labelling pattern was essentially unchanged. However, the FITC distribution on the gradient was markedly altered by this change in protocol. The total amount of FITC was increased 4–5-fold compared with treatment of intact cells, and the labelling pattern on the gradient more closely resembled the total-protein and endoplasmic-reticulum profiles than that of the plasma membrane.

DISCUSSION

We tested three models proposed for insulin stimulation of hexose transport (see the Models of insulin stimulation of glucose transport section) by measuring the fold increase above basal caused by maximal insulin stimulation over a range of FITC pretreatment concentrations. Fluorescein isothiocyanate irreversibly inhibits the transport of 2-deoxy-D-glucose in subsequently washed cells in a dose-dependent manner, with half-maximal inhibition occurring at approx. 2 mM-FITC at 37 °C in pH 9.2 buffer in 8 min.

Fig. 1 shows the experimental results for comparison with theoretical model curves generated from eqns. (9) and (10). The fold stimulation of transport above existing

(inhibited) basal rates is comparable at all FITC doses tested, and does not differ significantly from control fold increases caused by insulin, indicating that Model AI cannot account for the experimental data. This suggests that insulin does not activate 'latent' transporters within the membrane that are insensitive to FITC inhibition and that the insulin stimulation process does not involve insertion of transporters from inside the cell (not accessible to drug inhibition) into the membrane.

The strength of the above suggestion is dependent on the relative ability of FITC (1) selectively to inhibit surface transporters without corresponding effects on any transporters located at intracellular sites (Cushman & Wardzala, 1980), and (2) to allow the insulin stimulation process to proceed unimpeded. At pH 9.2, the pH at which the cells are incubated for irreversible modification, the permeability of FITC through the plasma membrane is likely to be low, owing to the poor lipid solubility of the ionized species (octanol/buffer partition coefficient $\approx 10^{-3}$ at pH 9.2). Although the intracellular milieu has been shown to be buffered to a certain degree against changes in extracellular pH (Piwnicka-Worms *et al.*, 1985) the capacity of this system in adipocytes is unknown.

To evaluate whether FITC treatment of intact cells could modify intracellular proteins, two different approaches were used. FITC treatment at pH 9.2 rapidly inactivates hexokinase from both adipocytes and yeast. After treatment of intact adipocytes with FITC, no change in endogenous hexokinase activity could be detected. These experiments establish that transport measurements are not complicated by measurable changes in hexokinase activity after FITC treatment, and also that intracellular conditions during FITC treatment of intact cells do not appear to favour modification of hexokinase. It was also established that FITC treatment had no effect on total intracellular ATP. This observation is crucial to evaluating insulin stimulation of 2-deoxyglucose uptake, an ATP-dependent mechanism, and also suggests that FITC treatment of intact cells did not disturb mitochondrial function.

In a second series of experiments, the subcellular distribution of FITC, after labelling of intact cells, closely paralleled the distribution of the plasma-membrane marker, ^{125}I -WGA, on the sucrose gradient, and was completely different from the distribution of markers for endoplasmic reticulum, Golgi, protein and mitochondria. An entirely different distribution of FITC on the gradient results when the adipocyte homogenate is directly labelled, more closely resembling the total protein distribution on the gradient. Under the latter conditions the sedimentation density profile of the plasma-membrane marker is only marginally affected. These studies demonstrate that, although the extent of labelling of intracellular organelles cannot be directly quantified (owing to the overlapping resolution of organelle material on the gradient), no evidence for labelling of cellular material other than the plasma membrane is apparent. Thus the extent of inhibition of surface membrane transporters by FITC would be expected to be in excess of inhibition of any intracellular transporters (protected from modification) that might be destined for expression on the cell surface in response to the hormone.

We cannot exclude the possibility that FITC labelling of some exofacial protein or lipid interferes with the process by which insulin activates transport, thereby complicating the above interpretation of the data.

Fig. 5. Subcellular distribution of organelle markers, total protein and FITC in isopycnic sucrose density gradients

The percentage of the total marker activity recovered on the gradient ($Q/\Sigma Q$) was plotted as a function of the sucrose concentration (% w/w). Total activities of marker enzymes, total protein and total FITC bound are listed in Table 2 and were measured as indicated in the text. Marker distributions are shown for: (a) total homogenate protein; (b) endoplasmic reticulum; (c) Golgi; (d) intact mitochondria; (e) and (h) plasma membrane. Panels (f) and (g) show gradient distributions of fluorescein.

However, the observation that the dose-response for hormone activation of hexose uptake in FITC-treated cells is unchanged suggests that such a possibility is unlikely. Also, labelling of plasma-membrane lipid components by exposure of intact cells to FITC seems unlikely, as over 97% of the amino phospholipids (phosphatidylethanolamine and phosphatidylserine) reside within the inner monolayer of the plasma membrane in adipocytes (Hyslop, 1981). If indeed transporter translocation was occurring in response to insulin, then from eqn. (9), when $\sigma'_{[AI]} = 2$, the product of the fractional inhibited transport rate and the concentration of basal carriers (iC_B) would have to be always exactly equal to the concentration of new carriers inserted into the plasma membrane (C_I) to be consistent with the observed constant stimulation. The mechanism of hexose permeation through the carrier and insulin activation of transport are clearly independent processes (Hyslop *et al.*, 1984a), and any inhibition of the latter is not likely to exhibit identical dose-dependency and rates of modification that are observed for transporter inhibition. Cushman and co-workers (Karnieli *et al.*, 1981) have suggested that insulin activates glucose transport by a two-step process, where translocated transporters are subsequently activated within the plasma membrane. Additional studies are necessary to determine whether FITC pretreatment could interfere with this membrane-activation step, thereby masking functional detection of any inserted transporters.

Early studies of the mechanism of action of insulin conducted with non-metabolizable carbohydrates lead to the conclusion that insulin increases the permeability of the plasma membrane to glucose (Morgan & Neely, 1972). Since models AII and B predict the observed responsiveness data exactly, we favour the view that the observed constant stimulation of hexose transport by insulin represents stimulation of the activity of transporters already in the plasma membrane. Indeed, a variety of membrane and cytoskeletal perturbants, such as alcohols, filipin (Sauerheber *et al.*, 1982; Hutchinson *et al.*, 1985) and cytochalasin B (Jarett & Smith, 1979) inhibit both basal and insulin-stimulated hexose transporters, but are without effect on the mechanism by which insulin activates hexose transport. This supports the concept that insulin stimulation of transport occurs via a process involving relatively close association of the insulin receptor and glucose transporter, perhaps analogous in part to allosteric regulation of a V-series enzyme (Martin *et al.*, 1981). In this regard, Jarett & Smith (1979) earlier proposed that the insulin receptor and the glucose-transport system might be physically associated or linked with a common glycoprotein. However, additional experiments are necessary, perhaps with other transport-inhibiting agents or with antibody to the transport system in the native membrane, to address this question further.

We thank the Rees-Stealy Research Foundation Board for assistance, Dr. Arne N. Wick for advice, Mariel Taraz for excellent secretarial help, and Dr. L. A. Sklar for use of the SLM 8000 spectrophotometer. Finally, we are especially indebted to Dr. Algirdas Jesaitis for both assistance and advice with the preparation of the sucrose gradients. These studies were supported in part by the National Institutes of Health (grant AM28431) and by the California Metabolic Research Foundation.

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Received 18 February 1985/21 May 1985; accepted 30 May 1985