Insulin-stimulated Glucose Transport in Rat Adipose Cells

MODULATION OF TRANSPORTER INTRINSIC ACTIVITY BY ISOPROTERENOL AND ADENOSINE*

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The mechanism of modulation of insulin-stimulated glucose transport activity in isolated rat adipose cells by lipolytic and antilipolytic agents has been examined. We have measured glucose transport activity in intact cells with 3-O-methylglucose and in plasma membranes from rat adipose tissue. The inhibition of glucose transport activity in intact adipocytes by isoproterenol was measured by cooling the cells during the routine procedure of the membrane preparation. Plasma membranes from these cells contained an unchanged number of transporters (31 ± 7, mean ± S.E., versus 31 ± 4 pmol/mg of protein in controls) but transported glucose at a reduced rate (19 ± 6 versus 48 ± 9 pmol/mg of protein/s). Conversely, incubation of intact cells in the presence of adenosine stimulated plasma membrane glucose transport activity compared to that in the absence of adenosine (44 ± 6 versus 36 ± 6 pmol/mg of protein/s). Kinetic studies of isoproterenol-inhibited glucose transport in plasma membranes revealed a 60% decrease in Vmax (2900 ± 350 versus 7200 ± 1000 pmol/mg of protein/s) and a small increase in Km (15.1 ± 1 versus 13.0 ± 0.6 mM). These data indicate that modifications of glucose transport activity produced by lipolytic and antilipolytic agents in intact adipose cells can be fully retained in plasma membranes isolated under appropriate conditions. Furthermore, the effects of these agents occur through a modification of the glucose transporter intrinsic activity.

Several investigators have shown that catecholamines inhibit insulin-stimulated glucose transport activity in isolated adipose cells by 50–75%, provided that the antagonizing effect of adenosine is prevented either by removal of endogenous adenosine (1–4) or treatment with Bordetella pertussis toxin (5). The molecular basis of this effect might be attributed either to a reversal of the translocation of glucose transporters as produced by insulin (6, 7) or to a change in the intrinsic activity of a constant number of transporters. Whereas initial experiments measuring glucose-inhibitable cytochalasin B binding sites indicated that catecholamines might operate by a combination of both mechanisms (4), further experiments under more stringently defined conditions which prevent accumulation of fatty acids have suggested that the modulation occurs at the level of transporter intrinsic activity (8). However, this suggestion could not be substantiated by preparation of plasma membranes with reduced transport activity.

This communication describes experiments designed to find conditions which would preserve the inhibitory effect of isoproterenol in a plasma membrane preparation. The data demonstrate that the effect of the catecholamine is reversed by cooling the cells during the routine procedure of the membrane preparation. However, the inhibition cannot be retained in the plasma membranes by use of a metabolic inhibitor (KCN). Since these membranes contain an unchanged number of glucose transporters, the results directly document a catecholamine-induced reduction in transporter intrinsic activity.

EXPERIMENTAL PROCEDURES

Animals and Cell Preparation—Male rats (CD strain, Charles River Breeding Laboratories), weighing 170–290 g, were used throughout. The animals were killed in the morning by cervical dislocation and decapitation, and the epididymal fat pads were removed. The adipose tissue was minced and digested with collagenase (Type 1, Cooper Biochemical) as described previously (9). All incubations were carried out at 37°C in a KRBH buffer, pH 7.4, containing 10 mM sodium bicarbonate, 30 mM Hepes, 5% bovine serum albumin (Fraction V, Reheis Chemical Co.), 2.5 mM glucose, and 200 mM adenosine, conditions developed by Honnor et al. (10, 11) to reduce fatty acid accumulation.

Determination of Glucose Transport Activity in Intact Cells—Glucose transport activity in adipose cells was assayed with 3-O-methylglucose (0.1 mM) under zero-trans conditions as previously described in detail (12).

Preparation of Plasma Membranes and Measurement of Glucose Transporter Concentration—The adipose cells obtained from 32 rats were incubated at 37°C in four polypropylene containers at a concentration of 0.8–1.2 x 10^6 cells/ml. According to the stringently defined conditions for lipolytic cells (10, 11), the shaking speed was adjusted to the minimum speed which would accomplish an even distribution of the cells in the medium (50 cycles/min for containers with a diameter of 12 cm containing a volume of 45 ml). After incubation as indicated with 10 mM insulin (crystalline zinc insulin, courtesy of Dr. R. E. Chance, Eli Lilly and Co.) and 2.5 μg/ml adenosine deaminase (from calf intestine, Boehringer Mannheim, Category 102121) either alone or in combination with 0.5 μM isoproterenol, KCN was added where indicated to a final concentration of 2 mM, and samples for the assay of glucose transport in the cells were taken. The remaining cells were washed once with TES homogenization buffer at 18°C, resuspended, and homogenized with a Potter-Elvejem grinder.

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1 The abbreviations used are: KRBH, Krebs-Ringer bicarbonate Hepes buffer; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, Tris-EDTA sucrose buffer.
Plasma membranes were prepared by differential centrifugation as previously described in detail (13). The concentration of glucose transporters in the membrane fractions was assessed using a specific D-glucose-inhibitable cytochalasin B binding assay as described (6).

Assay of Glucose Transport in Plasma Membranes—Glucose transport in plasma membrane vesicles was determined under equilibrium exchange conditions using a modification of the method of Ludvigsen and Jaret (14). Samples (20-40 μg) of membrane protein were incubated in 30 μl of TES buffer containing equal concentrations of D-glucose (0.1 mM in all experiments except in the kinetic studies) and L-glucose at 22°C for at least 30 min and were pulsed for 2.5 s with 30 μl of the same medium containing approximately 1 μCi of D-[U-14C]glucose and L-[3H]glucose. L-Glucose was present in order to correct for the noncarrier-mediated uptake and trapping. Uptake was stopped with a 15-fold dilution of ice-cold incubation medium containing 0.133 mM phloretin. The samples were filtered through a prewetted 0.22-μm Millipore membrane filter and washed three times with 2 ml of the ice-cold stopping solution. The filters were air-dried and transferred to 15-ml scintillation vials, and a toluene/xylene-based scintillation mixture was added. In order to determine the maximum uptake, samples were incubated for 30 min with labeled D-glucose. As was assessed in separate experiments, this time period was sufficient to achieve maximum uptake. The samples were stopped and filtered after addition of labeled L-glucose to correct for trapping. Initial velocities were calculated from the uptake values $U_t$ and $V_{\text{max}}$ as described previously (12). $K_a$ and $V_{\text{max}}$ were calculated by linear regression analysis of Hanes plots.

Calculations—All calculations were carried out on the Dartmouth Time-Shared System computer facilities. Statistical significance was tested with a paired $t$ test and was accepted at the $p \leq 0.05$ level.

RESULTS

Fig. 1 illustrates the effects of incubation temperature, cell washing, and KCN on the inhibition of insulin-stimulated glucose transport activity by isoproterenol in intact rat adipose cells. In cells which had been treated with a maximally stimulating insulin concentration (10 nM) for 25 min, a subsequent 15-min incubation with isoproterenol (0.5 μM) in combination with adenosine deaminase to remove adenosine reduced the rate of 3-O-methylglucose transport by approximately 60% as compared to the rate observed with adenosine removal alone. If cells were cooled to room temperature after the above described treatment and transport was assayed at that temperature, transport activity was lowered in controls (adenosine removal alone) but not in isoproterenol-treated cells. The temperature change thus resulted in a net loss of the inhibitory effect of isoproterenol. After washing the cells with the homogenization buffer, the effect of isoproterenol similarly disappeared, despite assaying transport activity in this part of the experiment after re-equilibration of the cells to 37°C. KCN (2 mM) added prior to cooling and washing the cells preserved the effect of isoproterenol. Although the absolute transport rates appeared to be lower in the washed cells than in those incubated in KRBH buffer, the magnitudes of the inhibitory effects of isoproterenol were comparable (approximately 60%).

A study of the time course of the reversal of isoproterenol’s inhibitory effect by cooling revealed that the effect was already lost 5 min after the temperature change (data not shown). KCN could block the reversing effect of temperature as well as the known reversal of the inhibitory effect of isoproterenol by a β-adrenoceptor antagonist (propranolol) and an adenosine receptor agonists (N’-phenylisopropyladenosine) (data not shown).

Table I shows the effects of isoproterenol and adenosine treatment of cells on insulin-stimulated glucose transport in intact cells and isolated plasma membranes prepared from these cells. KCN treatment of cells prior to washing and homogenization preserved the inhibitory effect of isoproterenol on transport activity measured in the isolated plasma membranes. Conversely, adenosine enhanced the insulin-stimulated transport measured in membranes by approximately 20% as compared to the absence of adenosine. As can be seen from Table II, the inhibitory effect of isoproterenol is largely due to a decrease in the transport $V_{\text{max}}$ (60%). However, a significant but small increase in $K_a$ was also observed.

Fig. 2 shows the effects of preparing plasma membranes from isoproterenol-treated cells in the presence and absence of KCN on glucose transporter concentration and transport activity. With or without KCN treatment the concentration of glucose transporters was unchanged as judged from the number of glucose-inhibitable cytochalasin B binding sites. However, isoproterenol moderately but significantly decreased the affinity of the cytochalasin binding site ($K_a = 135 \pm 20$ nM, mean ± S.E., versus $95 \pm 14$ without isoproterenol). As anticipated, plasma membranes obtained from isoproterenol-treated cells showed a significantly reduced glucose transport activity, provided that the cells had been treated with

### Table I

| Glucose transport activity in intact cells and plasma membranes from insulin-stimulated rat adipose cells treated with adenosine and isoproterenol |
|---|---|
| Isolated cells were incubated for 25 min at 37°C in the presence of adenosine (200 nM) without (basal) or with insulin (10 nM) as indicated. Isoproterenol (0.5 μM) and/or adenosine deaminase (2.5 μg/ml) were added (hatched bars) and the cells were incubated for another 15 min; controls received adenosine deaminase alone. Methanol-glycose was transported either immediately at 37°C, after cooling of cells to 22°C, after washing once and redistributing the cells in a volume of TES homogenization buffer equal to that of the original incubation buffer, or after the addition of KCN (2 mM in a series of samples from each of three separate experiments). | 
| 3-O-Methylglucose transport activity in plasma membranes (pmol/mg protein/s) | 
| Basal | 0.06 ± 0.01 | 4.7 ± 1.4 |
| Insulin | 1.61 ± 0.21 | 35.6 ± 6.0 |
| Insulin + adenosine | 2.34 ± 0.21 | 44.2 ± 6.0 |
| Insulin + isoproterenol | 0.83 ± 0.13 | 14.5 ± 3.0 |
of adenosine (200 nM) and insulin (10 nM) and thereafter for 15 min with adenosine deaminase (2.5 pg/ml) alone or in combination with isoproterenol (0.5 μM). The cells were fixed with KCN (2 mM), plasma membranes were isolated, and glucose transport was measured as described under "Experimental Procedures." Results are the mean ± S.E. of the single values obtained in each of three separate experiments.

|                | V_max (pN/mg protein/s) | K_m (mM) |
|----------------|-------------------------|----------|
| Insulin        | 7200 ± 1000             | 13.0 ± 0.6 |
| Insulin + isoproterenol | 2900 ± 350            | 15.1 ± 1.0 |

**Table II** Kinetic parameters of glucose transport in plasma membranes from insulin-stimulated rat adipose cells treated with isoproterenol

Isolated cells were incubated for 25 min at 37 °C in the presence of adenosine (200 nM) and insulin (10 nM) and thereafter for 15 min with adenosine deaminase (2.5 pg/ml) alone or in combination with isoproterenol (0.5 μM). The cells were fixed with KCN (2 mM), plasma membranes were isolated, and glucose transport was measured as described under "Experimental Procedures." Results are the mean ± S.E. of the single values obtained in each of three separate experiments.

**Fig. 2. Effects of isoproterenol on the relationship between insulin-stimulated glucose transport activity and glucose transporters in rat adipose cells.** Isolated cells were incubated with insulin and isoproterenol as described in the legend of Fig. 1. Where indicated, KCN was added 2 min before washing the cells with homogenization buffer. Methylnaphtholase in cells (A) was measured before washing, and D-glucose transport (B) and glucose transporter concentrations (C) were measured as described under "Experimental Procedures." Results are the mean ± S.E. of the single or mean values obtained in each of three separate experiments.

with KCN prior to homogenization. The correlation between the magnitudes of the effects in the cells and in the membranes was striking, both showing an approximately 60% inhibition. The isoproterenol effect was lost in cells which had been homogenized without prior fixation with KCN.

**Discussion**

The above presented data show that the inhibitory effect of catecholamines on insulin-stimulated glucose transport activity in adipose cells is lost during the routine procedure preceding homogenization and fractionation of these cells. We therefore studied in detail the reversibility of the inhibition of transport activity in order to find conditions which would prevent the loss of the effect during the fractionation procedure. KCN proved to be a suitable agent in blocking the reversibility of the effect during cooling and washing the cells with homogenization buffer, allowing the preparation of plasma membranes with inhibited transport activity (V_max) without any reduction in the number of glucose transporters. This finding clearly demonstrates that, unlike insulin, catecholamines modulate the intrinsic activity of the glucose transporter. In addition, it permits future experiments to define the structural changes involved in inhibiting transporter intrinsic activity as well as the underlying regulatory mechanisms.

In previous experiments the inhibitory effect of isoproterenol or the stimulatory action of adenosine, seen in the intact cell at 37 °C, could not be retained in isolated plasma membranes. During the routine preparation of plasma membranes,

the adipose cells were cooled and transferred from a KRBH buffer to a TES homogenization buffer prior to fractionation, and it is this cell cooling rather than the change in buffer that is responsible for the failure to retain these modulations in transport activity. In contrast to the rapid loss of the inhibitory isoproterenol effect seen here, cooling of the cells preserves insulin's stimulation of glucose transport activity by retarding the reversal of the translocation process (15, 16). These markedly different temperature dependencies infer different activation energies and consequently profound mechanistic differences of the two processes. Furthermore, KCN prevents translocation of transporters (15) but does not alter the transport activity already stimulated by insulin (17) (Fig. 1). It exerts, however, an effect on the isoproterenol/adenosine-mediated transport activity changes, further suggesting distinct mechanisms of the two regulatory processes.

The results presented in Fig. 2 demonstrate that isoproterenol does not change the concentration of plasma membrane glucose transporters, even when plasma membranes are prepared in the presence of KCN. This finding, together with the 60% reduction of plasma membrane glucose transport, represents the crucial piece of evidence for the conclusion that isoproterenol modulates the transporter intrinsic activity. It must be emphasized that the recently defined conditions for incubation of fat cells in the presence of lipolytic agents (10, 11), i.e. low cell concentration and adequate shaking speed, are essential for consistent measurements of glucose transporters using the cytochalasin B binding assay. In a previous study from this laboratory (4) not performed under the stringent conditions defined here, a significant reduction of cytochalasin binding sites was reported in response to isoproterenol treatment of adipocytes. Furthermore, insulin and isoproterenol had been added simultaneously. Therefore, differences in the experimental design might have allowed fatty acids to interfere with the cytochalasin B binding assay. Finally, since fatty acids have been reported to exert a moderate stimulation of glucose transport activity (18), they might even attenuate the inhibitory effect of catecholamines.

The kinetic data demonstrating a large decrease in V_max with only a small increase in K_m, agree with the characteristics of the catecholamine effect in the intact adipose cell (4). The inhibitory effect of the catecholamines can therefore be clearly distinguished from that of forskolin and isobutylmethylxanthine (5) and from the stimulatory effect of fatty acids (18) which comprise changes in V_max and K_m of similar magnitude.

Like the inhibitory effect of isoproterenol, the stimulatory effect of adenosine previously observed in intact adipose cells (4, 8, 19) could be preserved in the plasma membranes with the KCN treatment (Table I). Since the effect was small (20%) compared with the inhibitory effect of isoproterenol, we did not try to measure glucose transporters in the plasma membranes from adenosine-treated cells, anticipating that such a small difference would escape the low sensitivity of the cytochalasin B binding assay. It can be assumed, however, that isoproterenol and adenosine operate via closely related mechanisms as has previously been shown for the regulation of adenylate cyclase (20) and, more recently, for glucose transport activity itself (8). Accordingly, the inhibitory effect of isoproterenol is abolished in the presence of adenosine. Thus, the data suggest that an opposite regulation of the transporter intrinsic activity is mediated by the respective inhibitory and stimulatory GTP-binding regulatory proteins N_t and N_c.

The treatment of adipose cells with KCN in millimolar concentration lowers cellular ATP levels to less than 10% of normal (21), and prevents the development as well as the
reversal of the insulin effect on glucose transport (21, 22). Similar to this block of the recycling of glucose transporters, KCN prevents the translocation of insulin-like growth factor II receptors in response to insulin (17). The present finding that KCN treatment of cells preserved the effects of isoproterenol and adenosine in plasma membranes indicates that, like the insulin-regulated translocation, changes in intrinsic activity of the glucose transporter are energy-dependent. At present we can only speculate regarding the mechanisms which might account for such alterations. It remains to be determined whether a covalent structural change, possibly a phosphorylation/dephosphorylation reaction, could account for the energy dependence of the reversibility or whether a regulatory protein controlled by a nucleotide triphosphate could directly modulate the transporter intrinsic activity. A profound structural change of the transporter molecule might account for the observed effects, since isoproterenol altered the affinity of the cytochalasin B binding site of the glucose transporter.

A broad spectrum of alterations in glucose transport activity in insulin-resistant states appears to be sufficiently explained by alterations of the translocation of glucose transporter molecules from an intracellular pool to the plasma membrane (16). Very recently, however, several lines of evidence appeared suggesting long-term regulation of the intrinsic activity of the glucose transporter: recruitment of additional transporters could not fully account for the effects observed in insulin hyper-responsive states (23). However, attempts to demonstrate the change in intrinsic activity of the transporter failed, because the effects, as observed in the intact cells, apparently disappeared during preparation of plasma membranes. It remains to be elucidated, whether the observed effects can ultimately be defined as changes in transporter intrinsic activity with the aid of the KCN fixation described above.

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