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Differential sensitivity to guanine nucleotides of basal and insulin-stimulated glucose transporter activity reconstituted from adipocyte membrane fractions

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The effects of GTPγS on glucose transport activity reconstituted from adipocyte membrane fractions were studied in order to test the hypothesis that intrinsic activity changes of the insulin-sensitive glucose transporter may be mediated by guanine nucleotide-dependent mechanisms. GTPγS and GTP inhibited reconstituted glucose transport activity by 50% in membrane fractions from insulin-treated cells in a concentration-dependent manner; no inhibitory effect was observed in membrane fractions obtained from basal cells. GDP, GMP and guanosine were less effective than GTP, whereas the adenine nucleotides ATPγS and AMP failed to reduce the reconstituted transport activity. The data indicate that guanine nucleotides may modulate the activity of the adipocyte glucose transporter. Since the effect is dependent on treatment of cells with insulin, the hormone appears to induce a specific functional alteration of the glucose transporter.

Insulin; Glucose transport; Guanine nucleotide

1. INTRODUCTION

Insulin produces a rapid stimulation of glucose transport activity through re-distribution of transporters from an intracellular transporter pool to the plasma membrane [1,2]. In contrast, adenylate cyclase stimulators and inhibitors modulate the insulin-stimulated glucose transport through a change in the intrinsic activity rather than through alteration of the number of transporters in the plasma membrane [3,4]. Since the effects of catecholamines on glucose transport could be dissociated from those on the cAMP-dependent protein kinase [4], a hypothetical model of transport regulation has been proposed in which guanine nucleotide-binding proteins regulate transport activity by direct interaction with the transporter [4,5]. In the present study, we attempted to modulate the transport activity with guanine nucleotides in a cell-free system in order to test this hypothesis. The data show that guanine nucleotides, but not adenine nucleotides, inhibit transport activity reconstituted from both plasma membranes and intracellular low-density microsomes, provided that the cells had been exposed to insulin prior to the preparation of membranes. Thus insulin renders glucose transporters sensitive to guanine nucleotides, and might therefore control intrinsic activity changes of the glucose transporter mediated by guanine nucleotide-operated mechanisms.

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Abbreviations: KRBH, Krebs-Ringer-Hepes buffer; G_s, stimulatory guanine nucleotide-binding protein; G_i, inhibitory guanine nucleotide-binding protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Animals and cell preparation*

Male Wistar rats, weighing 160–220 g, were bred at our in-

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stitute. Adipose cells were isolated from epididymal adipose tissue by collagenase digestion (type 1, from Boehringer Mannheim GmbH, FRG) as described [6] with minor variations [7]. All incubations were carried out at 37°C in a KRBH buffer [7], pH 7.4, containing 4% bovine albumin (Fraction V, Serva Chemicals, Heidelberg, FRG), 1 mM glucose, and 200 nM adenosine.

2.2. *Preparation of plasma membranes and low-density microsomes*

Isolated adipocytes were homogenized as previously described in detail [8] in a buffer containing Tris (20 mM), sucrose (255 mM), and EDTA (1 mM). Plasma membranes and low-density microsomes were isolated by differential centrifugation as described [7]. The average cross-contamination of low-density microsomes as assessed with isoproterenol-stimulated adenylate cyclase activity was 5%. In each plasma membrane preparation, glucose transport activity in membrane vesicles was determined [8] as a control of the effect of insulin. Transport activity in membranes from insulin-treated cells was 8–10 times higher than in membranes from basal cells.

2.3. *Reconstitution of glucose transport activity*

Glucose transport activity was reconstituted into egg lecithin liposomes with the freeze-thaw technique essentially as described by Robinson et al. [9]. Briefly, membrane fractions were washed once with Tris buffer (20 mM, pH 7.4) to remove sucrose and EDTA, and were solubilized with 20 mM sodium cholate in Tris buffer (20 mM, pH 7.4) containing 1 mM magnesium chloride. Excess detergent was removed by gel filtration, and the transporter solution was combined with lecithin liposomes which had been prepared by two 3 min sonications of a 16% solution of egg lecithin. The power output of our sonicator (Branson, Model B12) was calibrated with glass beads, 125 mm in diameter (from Heat Systems Ultrasonics), as described [9], and all sonications of the liposomes were performed on setting 10. The samples were sonicated for 5 s, frozen at −80°C, thawed, and sonicated again for 12 s. GTPγS (Sigma Chemicals, St. Louis, MO, cat. no. G-8634) or the other nucleotides were added as indicated either before solubilization or after reconstitution. Transport was assayed at 37°C with D-[U-^{14}C]glucose, and rates were corrected for non-carrier mediated uptake with L-[1-^{3}H]glucose. The assay was stopped after 10 s, and samples were filtered on membrane filters ME 24 (Schleicher and Schüll, Dassel, FRG, cat. no. 401780). The dried filters were immersed in a water-compatible scintillation cocktail, and shaken for 2 h before liquid scintillation counting.

2.4. *Immunoblotting of G-protein α-subunits*

Samples of membrane fractions (40 μg of protein) were separated by SDS-PAGE and transferred to nitrocellulose sheets (Schleicher & Schüll, Dassel, FRG) with a semi-dry blotting apparatus (Sartoblott II, Sartorius, Göttingen, FRG). Sheets were blocked overnight by incubation with buffer containing 0.05% (w/v) Tween 20 and 150 mM sodium chloride, and were incubated with antiserum AS8 [10] against a peptide corresponding to a highly conserved region of G-protein α-subunits (α_{common}-peptide, sequence in one-letter code: (C)GAGESGKSTIVKQMK) at a dilution of 1:200 for 2 h at room temperature. After 3 washes with buffer containing albumin (1%), sheets were incubated with ^{125}I-protein A (0.1 μCi/ml) for 2 h, thoroughly washed, and autoradiographed for 1–4 days.

3. RESULTS

Treatment of isolated adipocytes with insulin has previously been shown to produce a redistribution of the reconstituted glucose transport activity from the low-density microsomes to the plasma membranes [2]. Confirming this finding, fig.1 shows that insulin increased the reconstituted D-glucose transport activity in plasma membranes, and decreased the transport activity in low-density microsomes. In addition, the figure illustrates the effects of GTPγS on glucose transport activity in the membrane fractions obtained from basal or insulin-treated cells. When plasma membrane glucose transporters were reconstituted in the presence of 500 μM GTPγS, transport activity was significantly reduced. In contrast, glucose transport reconstituted from basal plasma membranes was not affected by the nucleotide (fig.1, left panel). Similarly, when glucose transporters from low-density microsomes were reconstituted in the presence of 100 μM GTPγS (fig.1, left panel), the nucleotide produced a significant inhibition of the transport rate only in those prepared from insulin-treated cells. The non-carrier mediated glucose uptake into the vesicles (L-glucose uptake) was not altered by GTPγS (data not shown).

In the above described experiments (fig.1), GTPγS was added prior to the reconstitution procedure, since we were concerned that the highly charged molecule might not be able to penetrate the vesicles. In order to test whether the effect of GTPγS depended on its presence before and during the reconstitution procedure, control experiments were performed in which the nucleotide was added after the second sonication. Lecithin vesicles were incubated in the presence of GTPγS at 37°C for 10 min after reconstitution of plasma membrane glucose transporters. This treatment produced a 53% inhibition of D-glucose transport activity (0.68 ± 0.07 vs 1.45 ± 0.01 nmol/(mg protein × 10 s) in the controls, means ± SE of triplicate samples), similar to that observed when the nucleotide was present throughout the reconstitution procedure.

Fig.2 illustrates the data of experiments in which the concentration dependency of the effect of

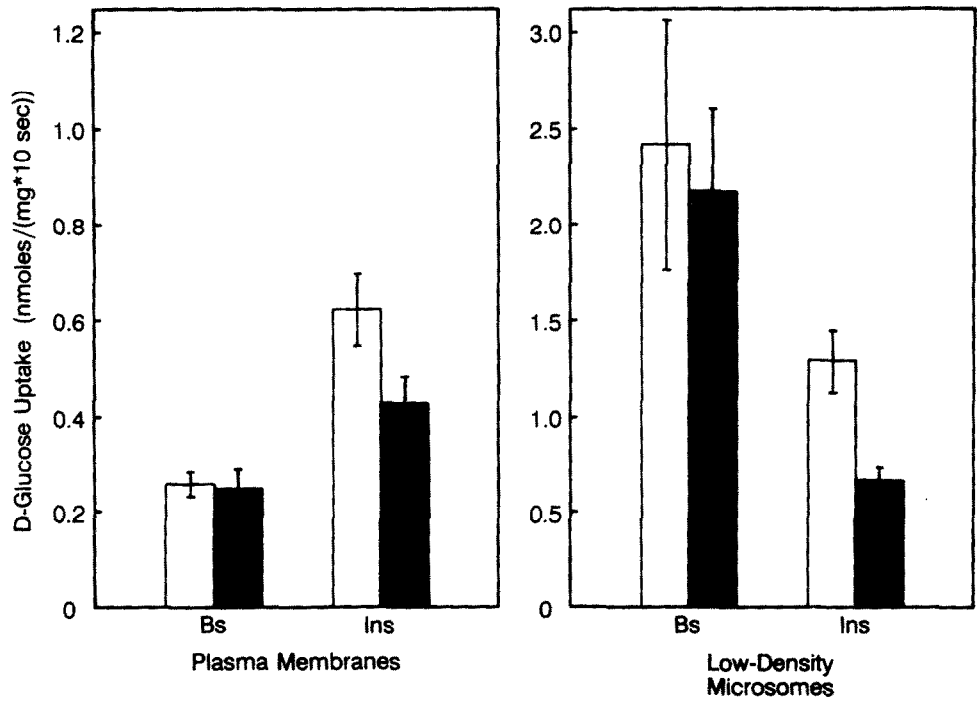

Fig.1. Effects of GTPγS on reconstituted glucose transport activity in plasma membranes and low-density microsomes from basal and insulin-treated adipocytes. Isolated adipocytes were incubated in the presence (Ins) or absence (Bs) of 8 nM insulin for 30 min, and membrane fractions were isolated. Plasma membranes (left panel) or low-density microsomes (right panel) were solubilized and reconstituted into egg lecithin liposomes in the presence (shaded bars) or absence (open bars) of 500 or 100 μM GTPγS, respectively. The data represent means ± SE of 3 independent experiments each performed with triplicate samples.

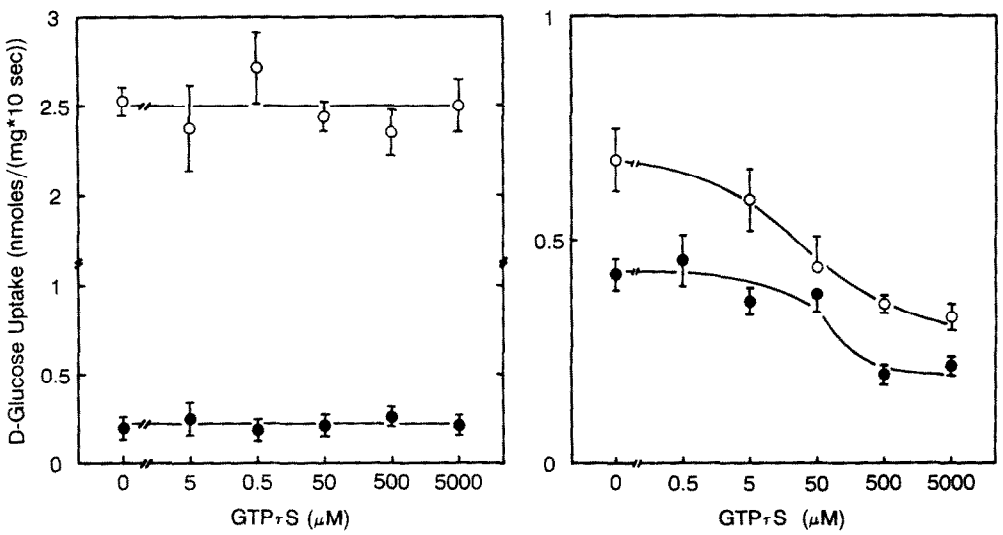

Fig.2. Concentration dependence of the effect of GTPγS on reconstituted glucose transport activity in plasma membranes and low-density microsomes from basal and insulin-treated adipocytes. Plasma membranes (filled circles) and low-density microsomes (open circles) were isolated from basal (left panel) or insulin-treated adipocytes (right panel). Membrane fractions were solubilized and reconstituted into egg lecithin liposomes with the freeze-thaw technique in the presence of the indicated concentrations of GTPγS, and stereospecific glucose uptake was assayed as described. The data represent means ± SE of triplicate samples from a representative experimental series.

GTPγS was studied. In plasma membranes and low-density microsomes from basal adipocytes, D-glucose transport activity was unaltered over the whole range of concentrations employed. In membrane fractions from insulin-treated adipocytes, GTPγS produced a concentration-dependent inhibition of the transport activity. Half-maximal inhibitory concentrations were in the micromolar range, and appeared to be higher for plasma membranes than for low-density microsomes. The maximal inhibitory effect was similar in both plasma membranes (52%) and low-density microsomes (58%).

Several adenine and guanine nucleotides were tested for their ability to mimic the inhibitory effect of GTPγS on reconstituted D-glucose transport activity from low-density microsomes (table 1). GTP (100 μM) produced a similar inhibition, whereas the inhibitory effects of GDP, GMP and guanosine were smaller, albeit statistically significant. The adenine nucleotides ATPγS and AMP failed to affect the reconstituted transport activity (table 1). Similar results (data not shown) were obtained in experiments with plasma membranes from insulin-stimulated adipocytes.

The above described data indicate that the inhibitory effect of the guanine nucleotides was not restricted to glucose transporters from plasma membranes, but was also observed when transporters were reconstituted from low-density microsomes. This finding prompted us to study the subcellular distribution of G-proteins in adipocytes, since participation of the stimulatory G-protein, G_s, in the effect of GTPγS would be ruled out easily, if no G-proteins were detected in the low-density microsomes. Fig.3 shows the immunoblots of plasma membranes and low-density microsomes obtained with an antiserum (AS8) against a highly conserved peptide sequence (α_{common}) of G-protein α-subunits [10,11]. The immunoreactive G_s and G_i α-subunits have previously been identified by comparison of their apparent

Table 1

Effects of various nucleotides on reconstituted glucose transport activity

Nucleotide	D-Glucose transport activity (% of controls)
GTPγS	49.1 ± 8.8
GTP	45.9 ± 8.4
GDP	69.3 ± 8.4
GMP	67.9 ± 9.3
Guanosine	74.8 ± 9.3
ATPγS	97.0 ± 1.5
AMP	102.8 ± 3.1

Low-density microsomes from insulin-treated adipocytes were solubilized and reconstituted as described in section 2 in the presence of 100 μM GTPγS, GTP, GDP, GMP, guanosine, AMP, or ATPγS as indicated. Data were normalized for control values obtained in the absence of added nucleotide, and represent means of at least 3 independent experiments performed in triplicate samples. Differences to the respective control values were significant ($p < 0.05$) for GTPγS, GTP, GDP, GMP and guanosine

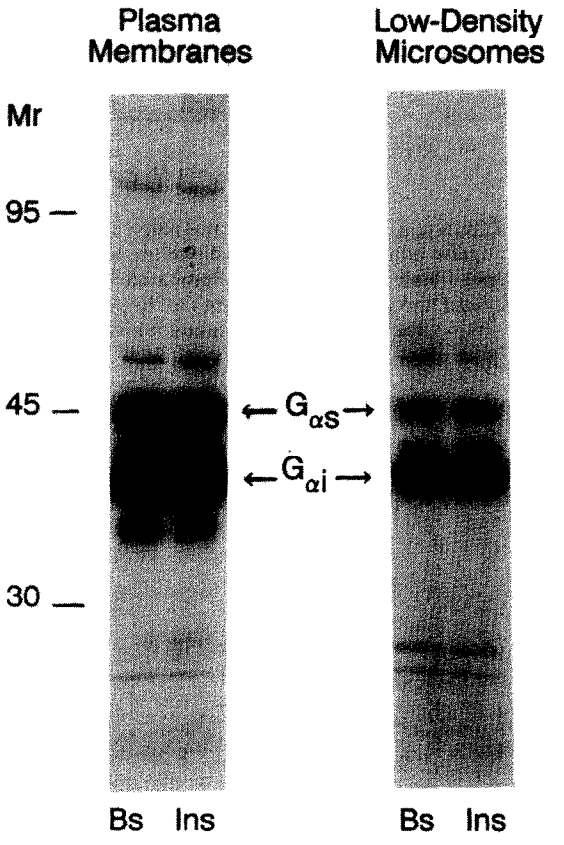

Fig.3. Western blot of G-protein α-subunits in membrane fractions from isolated adipocytes. Samples of 40 μg of protein from plasma membranes and low-density microsomes were separated by SDS gel electrophoresis (10% polyacrylamide gels) and immunoblotted as described with antiserum against a highly conserved peptide sequence (α_{common}) of G-protein α-subunits. Bs, membranes from basal cells; Ins, membranes from insulin-treated cells.

molecular weight with that of ADP-ribosylated α-subunits from adipocytes [10]. As is illustrated in the figure, both plasma membranes and low-density microsomes contained immunoreactive proteins at 45 kDa (α-subunit of G_s) and at 40 kDa (α-subunit of G_i). However, the immunoreactive G-protein α-subunits were much less abundant in the low-density microsomes than in the plasma membranes. Quantitation of the Western blots from 3 different sets of membranes revealed that the 45 and 40 kDa band (α-subunits of G_s and G_i) from low-density microsomes incorporated 9.5 ± 3.6 and 11.8 ± 3.7% (means ± SE), respectively, of the radioactivity found in the corresponding bands in plasma membranes. These values are close to the activity of isoproterenol-stimulated adenylate cyclase present in the LDM (5% of the activity in plasma membranes). Thus, cross-contamination of LDM with plasma membranes can account for most, if not all, of the G-proteins detected in the intracellular microsomes.

4. DISCUSSION

The above presented data indicate that guanine nucleotides inhibit glucose transport activity reconstituted from adipocyte membrane fractions. Guanine nucleotide-operated mechanisms might therefore be involved in the regulation of the intrinsic activity of glucose transporters. Most strikingly, the effect of the nucleotides depended on treatment of cells with insulin prior to the preparation of membranes, since even very large concentrations (5 mM) of GTPγS failed to reduce the D-glucose transport activity reconstituted from basal cells. Thus, the hormone appears to induce a specific functional alteration of the glucose transporter by rendering it sensitive to guanine nucleotides.

Heterogeneity of adipocyte glucose transporters, depending on their cellular localization and on the hormonal stimulation, has been reported in several previous studies. Radiation target size analysis of the transporter suggested that the low-density microsomal glucose transporter forms a dimer, since its molecular weight was about twice that of the transporter in plasma membranes [12]. Isoelectric focussing of the photolabeled transporter revealed two species with different isoelectric points, only one of which was translocated in response to insulin [13]. In addition, insulin appears to induce specific functional alterations of the transporter protein: the temperature dependence of glucose transport activity in plasma membranes from insulin-stimulated adipocytes differs from that in membranes of basal cells [8]. Further, insulin decreases the affinity of the cytochalasin B-binding site of glucose transporters in the low-density microsomes [14]. Like the above presented findings, these data strongly suggest that insulin initiates a structural and functional alteration of the transporter protein in addition to its translocation to the plasma membrane.

It has to be considered whether the observed effects have been produced through the previously proposed [4,5] interaction of G-proteins, namely G_s, with the glucose transporter. However, the above presented data do not unambiguously support such a mechanism. Firstly, the low-density microsomal glucose transporters appeared more sensitive to GTP. Although G-protein α-subunits were present in the low-density microsomes, possibly through cross-contamination of the microsomes with plasma membranes, their concentration was only 10% of that assayed in plasma membranes. Secondly, the concentrations of nucleotide required for the inhibition of transport activity were significantly higher than those required to modify adenylate cyclase activity in adipocyte membranes [15]. Thirdly, G-protein-mediated adenylate cyclase activation is selectively triggered by guanine triphosphates. In contrast, the observed effects were, although specific for guanine nucleotides, not restricted to the triphosphates, since GDP, GMP and guanosine produced similar, yet less pronounced effects. These clear differences between the effects observed and the regulation of adenylate cyclase argue against an interaction of G_s with the glucose transporter in analogy to that of G_s with adenylate cyclase. Alternatively, the involvement of other, yet unidentified G-protein species [16,17] or a direct effect of guanine nucleotides on a site at the transporter protein is conceivable.

In conclusion, the present data indicate that guanine nucleotides may modulate the activity of the insulin-sensitive glucose transporter. Since their effect is restricted to transporters from insulin-treated cells, the hormone appears to in-

duce a specific functional alteration of the transporter by rendering it sensitive to guanine nucleotides. The mechanism of such an insulin-dependent, guanine nucleotide-operated regulation of glucose transport remains to be elucidated.

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