Protein Synthesis Inhibitors Activate Glucose Transport without Increasing Plasma Membrane Glucose Transporters in 3T3-L1 Adipocytes*

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In this study, we tested the hypothesis that hexose transport regulation may involve proteins with relatively rapid turnover rates. 3T3-L1 adipocytes, which exhibit 10-fold increases in hexose transport rates within 30 min of the addition of 100 nM insulin, were utilized. Exposure of these cells to 300 μM anisomycin or 500 μM cycloheximide caused a maximal, 7-fold increase in 2-deoxyglucose transport rate after 4–8 h. The effects due to either insulin (0.5 h) or anisomycin (5 h) on the kinetics of zero-trans 3-O-methyl[14C]glucose transport were similar, resulting in 2.3±0.3-fold increases in apparent \( V_{\text{max}} \) values (control \( V_{\text{max}} = 1.6 ± 0.3 \times 10^{-7} \text{ mol/s/10^6 cells} \)) coupled with ~2-fold decreases in apparent \( K_m \) values (control \( K_m = 23 ± 3.3 \text{ mm} \)).

Insulin elicited the expected increases in plasma membrane levels of HepG2/erythrocyte (GLUT1) and muscle/adipocyte (GLUT4) transporters (1.6- and 2.8-fold, respectively) as determined by protein immunoblotting. In contrast, neither total cellular contents nor plasma membrane levels of these two transporter isoforms were increased when 3T3-L1 adipocytes were treated with either anisomycin or cycloheximide. 3-[\(^{125}\)I]-Iodo-4-azidoephethylamidio-7-O-succinyldeacetylflorskolin labeling of glucose transporters in plasma membrane fractions of similarly treated cells was also unaffected by these agents. Thus, a striking discrepancy was observed between the marked increase in cellular hexose transport rates due to these protein synthesis inhibitors and the unaltered amounts of glucose transporter proteins in the plasma membrane fraction. These data indicate that short-term protein synthesis inhibition in 3T3-L1 adipocytes leads to large increases in the intrinsic catalytic activity of one or both of the GLUT1 and GLUT4 transporter isoforms.

For most cell types, glucose transport is a vital process, directly affecting such metabolic pathways as glycogen and fatty acid synthesis. This is due to the fact that the rate of transmembrane glucose uptake often limits the rate of glucose metabolism. Thus, glucose transporter proteins serve as important regulatory elements in cellular metabolism. Of the hormones that influence glucose transport activity, insulin’s effect on glucose transport in cardiac and skeletal muscles and adipose tissues is the most extensively studied (1). The insulin-stimulated increase in glucose transport in fat and muscle cells is associated with a rapid redistribution of glucose transporter proteins from an intracellular pool to the plasma membrane, and these effects are complete within minutes of hormone addition (2–7). The elevated number of transporters in the plasma membrane of cells exposed to insulin is believed to contribute to the observed increase in glucose transport activity. Moreover, the insulin-stimulated redistribution of glucose transporters and insulin action on glucose uptake do not appear to be dependent upon new protein synthesis. Thus, for example, Kono et al. (8) showed that neither cycloheximide nor puromycin changes the insulin-stimulated redistribution of glucose transport activity reconstituted from either intracellular membranes or plasma membrane fractions derived from isolated rat adipocytes.

Recently, the role of protein synthesis in the regulation of the activity of glucose transporter proteins and their cellular distribution has become controversial. In two independent studies, Baly and Horuk (9) and Matthaei et al. (10) reported that cycloheximide-treated rat adipocytes exposed to insulin show no change in the amount of glucose transporters in the plasma membrane fraction compared to plasma membranes from control cells. Nevertheless, insulin is still able to elicit a maximal increase in glucose transport activity in intact rat adipocytes (9, 10) and in plasma membrane vesicles prepared from such adipocytes (9). Thus, it was concluded that the insulin-stimulated increase in glucose transport activity and glucose transporter redistribution to the plasma membrane fraction are dissociable events. These data suggested that insulin may increase the intrinsic catalytic activity of cell-surface transporters. However, these results were contradicted by Jones and Cushman (11), who reported that cycloheximide does not change the insulin-stimulated glucose transport activity or the distribution of glucose transporter proteins in isolated rat adipocytes. Therefore, they concluded that new protein synthesis is not required for the redistribution of glucose transporters to the cell surface or the associated increase in glucose transport activity in cells exposed to insulin.

The aim of this work was to evaluate whether inhibition of protein synthesis by anisomycin or cycloheximide regulates glucose transport activity or the subcellular distribution of either GLUT1 or GLUT4 protein in another insulin-respon-

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*This work was supported in part by Grant DK30898 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Recipient of a postdoctoral fellowship from the Juvenile Diabetes Foundation International.

§Supported by Postdoctoral Training Grant DK07302 from the National Institutes of Health.

1 GLUT1 and GLUT4 are designations for the HepG2/erythrocyte glucose transporter and the adult skeletal muscle/insulin-responsive glucose transporter, respectively, adapted from the nomenclature of Fukumoto et al. (48).
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Experimenlal Procedures

Materials

Cell Cultures—The 3T3-L1 fibroblasts used in this study were seeded at a density of 50 cells/mm² and grown as previously described (17). Briefly, fibroblasts were grown in DMEM containing 10% calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate and maintained in a 5% CO₂-humidified atmosphere at 37°C. Several days after the cells achieved confluence, differentiation was induced with DMEM containing 10% fetal bovine serum, 5 μg/ml insulin, 0.25 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate. After 2-3 days, the cells were exposed to the same medium without 3-isobutyl-1-methylxanthine and dexamethasone for an additional 2 days. Thereafter, the cells were maintained in DMEM containing 10% fetal bovine serum and antibiotics, and media changes were made at 2-3-day intervals. Cells were used 10-14 days after the induction of differentiation, at which time >95% of the cells expressed an apparent adipocyte phenotype that was determined by visual inspection of cell monolayers and without the aid of a microscope.

2-Deoxy-D-glucose Uptake Assays—Uptake in 3T3-L1 cells was measured using a modification of the method described by Frost and Lane (18). Briefly, cells were grown in 12-well culture plates and incubated in 1 ml of DMEM containing 10% fetal bovine serum ± 300 μM anisomycin or 500 μM cycloheximide for the times indicated. Following this preincubation period, cell monolayers were washed once with 1 ml of Krebs-Ringer phosphate buffer (170 mM NaCl, 3.3 mM KCl, 1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, pH 7.4), and subjected to centrifugation at 100,000 g, for 0.5 h. Plasma membranes were also resuspended in 6 ml of Buffer A to -1.5 mg of protein/ml. The material that sedimented at 250,000 g, for 1 h. Plasma membranes were removed from the top of the sucrose cushion, resuspended in 15-20 ml of Buffer B, and subjected to centrifugation at 30,000 x g for 0.5 h. Plasma membranes were resuspended in Buffer B to ~1.5 mg of protein/ml. The supernatant from the centrifugation at 250,000 x g was subjected to centrifugation at 30,000 x g for 1.5 h to collect the
low density microsomes, which were resuspended in Buffer B (towards 3–4 mg of protein/ml. All final membrane suspensions included 1 mM phenylmethylsulfonyl fluoride. The activity of 5'-nucleotidase (EC 3.1.3.5) was determined with a modification of the method described by Avruch and Wallach (20) as described previously (17).

**GLUT1 Proteins**—A peptide (TELE- YLGDPDEN, corresponding to amino acids 498–509 of rat GLUT4 (13) was synthesized (Hempeptide Systems, San Diego, CA) and coupled to keyhole limpet hemocyanin with glutaraldehyde (21). Rabbits were immunized (East European Biologicals, Southbridge, MA) with the peptide-keyhole limpet hemocyanin conjugate as described by Okruch et al. (14). Rabbits polyclonally antibodies selective for the non-transport domain of GLUT1 were generated as described previously (17).

- **[H]Leucine Incorporation into Protein—**3T3-L1 adipocytes, grown in 12-well culture plates, were incubated with 1 mM of serum-free DMEM ± 200 µM anisomycin or 500 µM cycloheximide for 1, 2, or 5 h at 37 °C. Following this incubation period, the medium in each well was removed, and the cells were then exposed to 0.5 ml of the same medium containing [H]leucine (2 µCi/ml ± anisomycin or cycloheximide as indicated for 1 h at 37 °C). Next, the cells were washed three times with 1 ml of ice-cold Krebs-Ringer phosphate buffer. Nonspecific uptake was defined by a 1-min exposure of cells to the medium containing [H]leucine before washing with ice-cold Krebs-Ringer phosphate buffer. The cells were incubated at 37 °C for 3 h in transfer buffer (150 mM NaCl, 20 mM Tris, 20% methanol, pH 8.2). Duplicate nitrocellulose filters were then transferred to nitrocellulose filters in duplicate filters-

- **Analysis of Glucose Transporter Proteins in Subcellular Membranes—**For each experiment, aliquots of subcellular membrane fractions containing equal amounts of total protein were subjected to SDS-10% polyacrylamide gel electrophoresis. In duplicate gels, under reducing conditions as described by Laemmli (22).Resolved proteins were then electroblotted to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band). The gels were then electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band). The gels were then electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band).

- **SDS-PAGE**—A standard procedure was followed with either 8% or 12.5% gels. The gels were then exposed to x-ray film (Kodak X-Omat film) at -70 °C with a Du Pont Lightning Plus film processor (Du Pont, Boston, MA).

- **Autoradiography**—A standard procedure was followed by the use of a X-Omat film (Kodak) at -70 °C with a Du Pont Lightning Plus film processor (Du Pont, Boston, MA). The gels were then exposed to x-ray film (Kodak X-Omat film) at -70 °C with a Du Pont Lightning Plus film processor (Du Pont, Boston, MA). The gels were then exposed to x-ray film (Kodak X-Omat film) at -70 °C with a Du Pont Lightning Plus film processor (Du Pont, Boston, MA).

- **[H]Glucose Incorporation into Glucose Transporter Proteins—**Membrane suspensions containing equal amounts of total protein were incubated for 1 h with 1 µCi of [H]glucose (2-3 H.5 M in ethanol) in Buffer B. Membrane proteins were precipitated by the addition of 0.15 M NaOH, and the cellular suspensions were centrifuged for 1 h at 37 °C. Following this centrifugation period, the medium in each well was removed, and the cells were then exposed to 0.5 ml of the same medium containing [H]leucine before washing with ice-cold Krebs-Ringer phosphate buffer. Nonspecific uptake was defined by a 1-min exposure of cells to the medium containing [H]leucine before washing with ice-cold Krebs-Ringer phosphate buffer. The cells were then electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band). The gels were then electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band). The gels were then electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band). The gels were then electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band). The gels were then electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell, pore size of 0.45 µm) at 200 mA for 3 h in transfer buffer (250 mM NaCl, 20 mM Tris, 0.5% bovine serum albumin, 0.5% gelatin, 0.1% Tween 20, 0.05% sodium azide, pH 7.5) for 1.5 h at room temperature. The buffer was then replaced with washing solution for the antibody and protein A solutions (composition is described above or below the transporter band).

**RESULTS**

**Effects of Insulin, Anisomycin, and Cycloheximide on Glucose Transport Activity—**It has been reported (17) that 100 nM insulin elicited a rapid (within 30 min, 10–15-fold increase in 2-deoxy-[H]glucose uptake over that observed in control 3T3-L1 adipocytes. Similarly, when 3T3-L1 adipocytes were treated with 300 µM anisomycin or 500 µM cycloheximide, there is a large increase in 2-deoxy-[H]glucose uptake (Fig. 1). However, in contrast to the rapid rate of onset of insulin action, the stimulatory effects of anisomycin or cycloheximide on hexose uptake develop more slowly. Although the time course for the effect due to anisomycin is somewhat more rapid than that due to cycloheximide, both agents gradually increase 2-deoxy-[H]glucose uptake until a maximal, 7-fold effect is achieved after 8 h. It should be noted that 40 µM cytochalasin B inhibits 2-deoxy-[H]glucose uptake in anisomycin- or cycloheximide-treated 3T3-L1 adipocytes by -96% (data not shown).

The cellular uptake of 2-deoxy-[H]glucose is the biochemical result of two tightly coupled events, i.e. substrate transport into the cell and its phosphorylation by hexokinase. Therefore, transport of 3-O-methyl-[14C]glucose (a nonmetabolizable substrate) into 3T3-L1 adipocytes was measured to specifically assess hexose transport. As shown in Fig. 2, 100 nM insulin elicited an almost 10-fold increase in the rate of 3-O-methyl-[14C]glucose transport. By comparison, cells treated with either 300 µM anisomycin or 500 µM cycloheximide for 5 h exhibit 8- and 5-fold increases, respectively, in 3-O-methyl-[14C]glucose transport. When cells are treated with both 300 µM anisomycin (5 h) and 100 nM insulin (0.5 h), the observed increase in hexose transport is greater than that elicited by either stimulant alone, but this effect is not completely additive. In contrast, the combination of 100 nM insulin and 500 µM cycloheximide produces an effect on

![Fig. 1. Time courses for effects of anisomycin and cycloheximide on 2-deoxy-[H]glucose uptake into 3T3-L1 adipocytes. Cells, grown in 12-well culture plates, were treated with 300 µM anisomycin or 500 µM cycloheximide at 37 °C for the times indicated. Uptake of 2-deoxy-[H]glucose was measured as described under "Experimental Procedures." The values shown for specific 2-deoxy-[H]glucose uptake were derived by subtracting out the nonspecific uptake that occurred in the presence of 40 µM cytochalasin B. The results presented are the means ± S.E. of triplicate determinations from four experiments.](https://example.com/fig1.png)
Effects of insulin, anisomycin, and cycloheximide on 3-O-methyl[14C]glucose transport into 3T3-L1 adipocytes. Cells grown in 12-well culture plates were treated with either 300 μM anisomycin (A) or 500 μM cycloheximide (C) for 5 h at 37 °C. Where indicated, cells were also treated with 100 nM insulin (Ins.) for 0.5 h at 37 °C. Transport assays were initiated with the addition of 3-O-methyl[14C]glucose to a final concentration of 50 μM as described under "Experimental Procedures." The values shown for specific 3-O-methyl[14C]glucose transport were derived by subtracting out the nonspecific uptake that occurred in the presence of 40 μM cytochalasin B. The results presented are the means ± S.E. of triplicate determinations from three experiments.

hexose transport that is no greater than that produced by insulin alone. It should be noted that compared to 100 nM insulin, treatment of 3T3-L1 adipocytes with either 300 μM anisomycin or 500 μM cycloheximide for 5 h does not affect intracellular volumes (~5-6 μl/10^6 cells) as measured by the accumulation of 3-O-methyl[14C]glucose at 4 h and 37 °C (data not shown).

The marked increase in hexose transport activity in 3T3-L1 adipocytes treated with insulin or anisomycin was analyzed as a function of the concentration of 3-O-methyl[14C]glucose. A representative experiment is shown in Fig. 3. 3-O-methyl[14C]glucose transport rates in cells treated with or without insulin or anisomycin displayed saturation kinetics. Apparent K_m and V_max parameters were determined by nonlinear regression analysis of the velocity versus [S] data using the equation: y = m1*m0/(m2 + m0), where m0 = [S], m1 = apparent V_max, and m2 = apparent K_m. This analysis assumes that transport exhibits simple hyperbolic kinetics. Table I shows these calculated values. For control cells, the apparent

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<thead>
<tr>
<th>Treatment</th>
<th>Apparent K_m (μM)</th>
<th>Apparent V_max (nmol/mg protein/min)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>200</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Insulin (n = 5)</td>
<td>100 nM insulin</td>
<td>4.1 ± 0.9</td>
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<tr>
<td>Anisomycin (n = 9)</td>
<td>100 nM anisomycin</td>
<td>4.6 ± 0.8</td>
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*These data are compiled from the results of four experiments performed during the course of the study and from the results of five experiments performed during the course of the study detailed in Footnote 3.

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K_m and V_max values for zero-trans influx of 3-O-methyl[14C]glucose are 23.3 ± 3.3 μM and 1.6 ± 0.3 × 10^{-2} nmol/mg protein/min, respectively (n = 8). When 3T3-L1 adipocytes are treated with 100 nM insulin for 30 min, the apparent K_m and V_max values are 10.9 ± 1.2 μM and 4.6 ± 0.8 × 10^{-2} nmol/mg protein/min, respectively (n = 9). Finallly, when cells are treated with 300 μM anisomycin for 5 h, the apparent K_m and V_max values for 3-O-methylglucose transport are 10.3 ± 1.9 μM and 4.1 ± 0.9 × 10^{-2} nmol/mg protein/min, respectively (n = 5). Thus, it appears that the increases in hexose transport rates caused by the actions of insulin or anisomycin in 3T3-L1 adipocytes are due to similar changes in both the apparent K_m and V_max values of hexose transport.

Effects of Anisomycin and Cycloheximide on Cellular and Plasma Membrane Glucose Transporter Protein Levels—Differentiated 3T3-L1 adipocytes were treated with 300 μM anisomycin or 500 μM cycloheximide for 5 h at 37 °C. The cells were then homogenized, and total cellular membranes were prepared to examine the effects of these agents on the relative amounts of GLUT1 and GLUT4 proteins in this membrane fraction. GLUT1 and GLUT4 proteins were detected with isoform-specific polyclonal antibodies raised against peptides corresponding to the C-terminal domains of each transporter protein. As shown in a representative protein immunoblot (Fig. 4A), treatment of 3T3-L1 adipocytes with anisomycin or cycloheximide has little if any effect on the amounts of either GLUT1 or GLUT4 protein in total cellular membranes. Immunoblots from three separate experiments were quantitated by excising and counting the activity in the relevant protein bands. The relative amounts of GLUT1 are 1, 0.99 ± 0.01, and 1.27 ± 0.35 for control and anisomycin- and cycloheximide-treated adipocytes, respectively. The small decrease in the amount of GLUT1 protein in total membranes from cells treated with anisomycin is statistically significant (paired t test, 0.01 < p < 0.02). Similarly, the relative amounts of GLUT4 protein are 1, 0.88 ± 0.06, and 1.05 ± 0.18 for control and anisomycin- and cycloheximide-treated adipocytes, respectively. However, the small decrease in the amount of GLUT4 protein in total membranes from cells treated with anisomycin is not statistically significant (p > 0.05).

To understand if the stimulatory effects of 300 μM anisomycin and 500 μM cycloheximide on hexose transport were
Anisomycin and Cycloheximide Increase Glucose Transport

Effects of Anisomycin on Insulin-stimulated Redistribution of Glucose Transporter Proteins—Experiments were performed to examine the ability of insulin to redistribute GLUT1 and GLUT4 proteins from the low density microsomal fraction to the plasma membrane fraction in 3T3-L1 adipocytes pretreated with anisomycin. A comparison of the effects of 100 nM insulin with and without prior treatment of cells with 300 µM anisomycin on the amounts of GLUT1 and GLUT4 proteins in low density microsomal and plasma membrane fractions is shown in a representative experiment (Fig. 5). As expected, treatment of 3T3-L1 cells with insulin alone decreased the relative amounts of GLUT1 and GLUT4 by 45 and 30%, respectively, in the low density microsomal fraction when compared to controls. This effect is associated with related to changes in the amounts of glucose transporters in the plasma membrane fraction, the relative amounts of GLUT1 and GLUT4 proteins in this membrane fraction were also examined (Fig. 4B). In these experiments, 3T3-L1 adipocytes were treated with anisomycin or cycloheximide for 5 h at 37 °C before cell homogenization and purification of the plasma membrane fraction. Immunoblots from three separate experiments were quantitated by excising and counting the relevant protein bands. The relative amounts of GLUT1 in the plasma membrane fraction are 1, 1.0 ± 0.1, and 1.1 ± 0.1 for control and anisomycin- and cycloheximide-treated adipocytes, respectively. The relative amounts of GLUT4 in the plasma membrane fraction are 1, 0.9 ± 0.2, and 1.1 ± 0.1 for control and anisomycin- and cycloheximide-treated cells, respectively. Similarly, no changes in the levels of GLUT1 or GLUT4 protein are observed in the low density microsomal fractions of cells treated with anisomycin or cycloheximide (data not shown in this series of experiments).

The abilities of anisomycin and cycloheximide to inhibit protein synthesis in 3T3-L1 adipocytes were also assessed (data not shown). Cells exposed to 300 µM anisomycin or 500 µM cycloheximide for 1 h before the addition of [3H]leucine exhibited 96 ± 3 and 88 ± 8% decreases, respectively, in [3H] leucine incorporation into trichloroacetic acid-insoluble material. Treatment of cells with either agent for 2 or 5 h before the addition of labeled leucine resulted in similar 96 ± 2–3% inhibitions of protein synthesis. The data demonstrate the virtual abolition of protein synthesis by anisomycin and cycloheximide under the conditions of our experiments.

It should be noted, however, that neither anisomycin nor cycloheximide appears to affect the amounts of total protein recovered in any of the subcellular membrane fractions from 3T3-L1 adipocytes. Approximately 3.2, 0.25, and 0.7 mg of total protein/150 × 20-mm culture plate are recovered for total, plasma, and low density microsomal membranes, respectively, for control or treated cells. The time course for the inhibition of [3H]leucine incorporation into new protein by 300 µM anisomycin and 500 µM cycloheximide was examined in two experiments. Anisomycin inhibited protein synthesis by 96 ± 3% after 1, 2, and 5 h. Similarly, the percent inhibition of protein synthesis by cycloheximide was 96 ± 2% after 2 and 5 h. However, the effect of cycloheximide after 1 h was more variable, being 88 ± 8%. In addition, none of the experimental conditions appear to alter the purity of the subcellular membrane fractions as assessed by the activity of 5'-nucleotidase. Thus, the specific activity of plasma membrane 5'-nucleotidase activity was always 6–8 times higher than that of low density microsomes, irrespective of anisomycin, cycloheximide, or insulin treatment of 3T3-L1 cells.

**Fig. 4.** Protein immunoblot analyses of effects of anisomycin and cycloheximide on amounts of GLUT1 and GLUT4 proteins in total cellular membranes and plasma membrane fractions from 3T3-L1 adipocytes. Cells, grown in 150 × 20-mm culture plates, were treated with 300 µM anisomycin (A) or 500 µM cycloheximide (C) for 5 h at 37 °C. Cells were homogenized, and total cellular membranes and plasma membrane fractions were prepared as described under “Experimental Procedures.” Total membrane proteins (50 µg) (A) or plasma membranes (50 µg) (B), from a representative experiment, were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis in duplicate gels and transferred to nitrocellulose filters. Immunoblot analyses were performed with antisera R-480 (anti-GLUT1) and R-1288 (anti-GLUT4) as described under “Experimental Procedures.” Immunoreactivity was quantitated by excising rectangular strips, corresponding to the locations of either GLUT1 or GLUT4 protein on the developed films, from the nitrocellulose filters and counting the filter strips. Specific binding of 125I-labeled protein A was determined after subtracting background radioactivity. Specific binding (in counts/minute) to GLUT1 total membranes was 434, 373, and 806 for control membranes or membranes from cells exposed to anisomycin or cycloheximide, respectively. Similarly, specific binding to GLUT4 total membranes was 1885, 1882, and 2266, respectively. Specific binding to GLUT1 and GLUT4 plasma membrane was 784, 643, and 671 and 1986, 1107, and 1702, respectively.

**Fig. 5.** Distributions of GLUT1 and GLUT4 proteins in plasma membrane and low density microsomal fractions obtained from 3T3-L1 adipocytes. Cells, grown in 150 × 20-mm culture plates, were treated with 300 µM anisomycin for 5 h at 37 °C. Where indicated, cells were also treated with 100 nM insulin (Ins.) for 0.5 h at 37 °C. The cells were homogenized, and plasma membrane (PM) and low density microsomal (LDM) fractions were prepared as described under “Experimental Procedures.” Membrane proteins (50 µg) were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Immunoblot analyses were performed with antisera R-480 (anti-GLUT1) and R-1288 (anti-GLUT4). Shown are the relevant strips of a representative Western blot.
Anisomycin and Cycloheximide Increase Glucose Transport

Anisomycin and cycloheximide increased glucose transport rates among all experimental conditions examined in Fig. 6. In 3T3-L1 cells treated with 100 nM insulin for 0.5 h, there is a 9.5-fold increase in hexose transport rate that is associated with a significant increase in the amounts of GLUT1 (1.6-fold) and GLUT4 (2.8-fold) proteins in the plasma membrane fraction. Similarly, when cells are exposed to either 300 μM anisomycin or 500 μM cycloheximide for 5 h, there are 7.3- and 4.8-fold increases in hexose transport, respectively. However, these effects on transport rates are not associated with any significant changes in the amounts of GLUT1 and GLUT4 proteins in the plasma membrane fraction when compared to control cells. In addition, treatment of cells with both insulin and anisomycin causes a 14-fold increase in hexose transport rates despite the observation that there are actually fewer glucose transporter proteins in the plasma membrane fraction compared to cells exposed only to insulin.

Due to the isoform selectivity of our antibodies, the above data do not exclude the possibility that one or more additional glucose transporter isoforms may contribute to glucose transport in 3T3-L1 adipocytes. If so, it is possible that exposure of these cells to anisomycin or cycloheximide could significantly increase the amount of such hypothetical transporters in the plasma membrane. To examine this hypothesis, glucose transporters present in the plasma membrane fractions of 3T3-L1 adipocytes were labeled with the photoaffinity ligand [125I]APS-forskolin. This reagent is expected to label all glucose transporter isoforms that might be present in a membrane fraction. The results from a representative experiment are shown in Fig. 7A. Quantitative analysis of two similar experiments is presented in Fig. 7B. As expected, the binding of [125I]APS-forskolin is increased in the plasma membrane fraction of anisomycin-treated cells compared to control cells. The increased binding is consistent with the observed increase in glucose transport rates.
of [125I]lAP5-forskolin to the glucose transporters (43-55-kDa band) in the plasma membrane fraction is substantially inhibited by cytochalasin B (~50% in these experiments). When cells are treated with 100 nM insulin for 30 min, there is a 1.95 ± 0.15-fold increase in labeling of the glucose transporter band. In contrast, there is no significant change in the amount of [125I]lAP5-forskolin labeling of glucose transporter proteins in the plasma membrane fraction from cells treated with either 300 μM anisomycin (1.15 ± 0.15-fold) or 500 μM cycloheximide (0.9 ± 0.1-fold) for 5 h. Treatment of cells with both insulin and anisomycin results in a 1.65 ± 0.35-fold increase in transporter labeling, which is an intermediate increase compared to the amount of labeling observed with either agent alone. In summary, these results are quantitatively similar to the immunoblot results shown in Fig. 6. Thus, these data obtained with [125I]lAP5-forskolin do not support the possibility that other glucose transporter isoforms are responsible for the increase in glucose transport induced by anisomycin and cycloheximide in 3T3-L1 adipocytes.

**DISCUSSION**

The results presented here demonstrate a marked stimulatory effect of 300 μM anisomycin and 500 μM cycloheximide on hexose transport in differentiated 3T3-L1 cells in culture. Similar effects are observed when either 2-deoxy[3H]glucose uptake (Fig. 1) or 3-O-methyl[3H]glucose transport (Fig. 2) rates are measured. However, the onset of the increase in hexose transport elicited by anisomycin and cycloheximide is considerably slower than that elicited by the action of 100 nM insulin. Whereas the increase in hexose transport due to insulin action is maximal within 30 min, transport in cells treated with anisomycin or cycloheximide increases to its maximal rates after 4-8 h (Fig. 1). Although both agents are slower in their rate of action than insulin, there are also differences in the time courses for the increases in glucose transport elicited by anisomycin versus cycloheximide. The onset of the increase in hexose transport in cells treated with anisomycin is significantly more rapid than that observed when cells are treated with cycloheximide. The reason for this difference is not clear. It is possible that these different time courses may be related to observations that anisomycin is a more stringent inhibitor of protein synthesis than cycloheximide (25, 26). Our data do not show any difference in the maximal percent inhibition of new protein synthesis by either reagent when added 2 or 5 h prior to labeled amino acid, but the inhibitory effect of anisomycin may be more rapid than that due to cycloheximide.

Despite differences in the onset of the increase in hexose transport elicited by insulin and anisomycin, both agents appear to have similar effects on the transport process in 3T3-L1 adipocytes (Fig. 3). Analyses of the effect of insulin on 3-O-methyl[3H]glucose transport as a function of substrate concentration show that insulin action increases the Vmax of transport 2.9-fold (from 1.6 to 4.6 × 10−7 mmol/s/106 cells). This effect of insulin qualitatively agrees with increases in Vmax observed in isolated rat adipocytes (27–30). In addition to an effect on Vmax, insulin action also decreases the apparent Km of transport 2.1-fold (from 23.3 to 10.9 mM). This finding is consistent with recent studies on isolated rat adipocytes in which the Km value for 3-O-methylglucose transport decreased ~2-fold (from 9.7 to 4.8 mM (27) and 19.6 to 7.1 mM (28)) upon treatment of cells with 10 nM insulin. However, it should be noted that the effect of insulin on the apparent Km of glucose transport in isolated rat adipocytes is controversial since no such effect of insulin was observed in other similar studies (29, 30). Like insulin, the action of anisomycin increases Vmax 2.5-fold, and this is coupled with a 2.2-fold decrease in the apparent Km. Despite these similarities in the effects of insulin and anisomycin on the apparent Km and Vmax values, for 3-O-methylglucose transport in 3T3-L1 adipocytes, these two stimulants may not have the same mechanism of action. This is due to the fact that the Km and Vmax values represent aggregate constants for the cellular transport process, which is composed of at least two glucose transporter isoforms. Therefore, this kinetic analysis does not provide detailed information about the effects of insulin and anisomycin on Km and Vmax parameters of transport mediated by the individual GLUT1 and GLUT4 proteins.

Our results on the actions of the protein synthesis inhibitors anisomycin and cycloheximide in 3T3-L1 adipocytes differ from those of earlier investigations (9, 10) on the effect of cycloheximide on glucose transport activity in isolated rat adipocytes. In those studies, rat adipocytes were treated with 10 μg/ml (36 μM) cycloheximide for 1.5 or 2 h (Refs. 10 and 9, respectively) without a detectable effect on glucose transport activity. It was only when insulin was added to such cells that an apparent increase in intrinsic catalytic activity of transporters was observed. However, Jones and Cushman (11) did observe that a 2-h exposure of rat adipocytes to 10 μg/ml cycloheximide results in a statistically significant 2-fold increase in glucose transport activity. Similarly, we also observed a ~2-fold increase in the rate of 2-deoxyglucose uptake when 3T3-L1 adipocytes were treated with 500 μM (141 μg/ml) cycloheximide for 2 h (Fig. 1). Moreover, when the time of treatment of 3T3-L1 cells with cycloheximide was extended, there was an additional increase in glucose transport activity (Fig. 1). One possible explanation for the conflicting reports (9–11) on the effect of cycloheximide on glucose transport activity in rat adipocytes may be that a 1.5–2-h treatment is the threshold time for an effect on transport. In support of this possibility, chronic treatment of NIL hamster cells with 300 μg/ml cycloheximide results in an increase in hexose transport (31).

Recent studies have demonstrated that changes in glucose transport activity directly correlate with changes in the total cellular amounts of either GLUT1 or GLUT4 protein when cells are chronically treated with agents such as insulin or tolbutamide (32), cholesterin or dibutyryl cAMP (17), and streptozocin (33, 34) or if cells are deprived of glucose (35). Therefore, it seemed possible that the increase in glucose transport activity in 3T3-L1 adipocytes treated with either protein synthesis inhibitor could be due to an increase in the total cellular amounts of one or both transporter isoforms. This may occur indirectly if the inhibition of protein synthesis resulted in a decrease in the turnover rates for the glucose transporters, thereby leading to their accumulation in the plasma membrane. However, treatment of 3T3-L1 adipocytes with anisomycin or cycloheximide for up to 5 h had little or no significant effect on the total cellular amounts of either GLUT1 or GLUT4 protein (Fig. 4A). These data suggest that turnover of the glucose transporter proteins in differentiated 3T3-L1 cells is slow when cells are treated with high concentrations of protein synthesis inhibitors. Similar findings were reported in a study of chicken embryo fibroblasts (36) in which the authors concluded that low concentrations (0.5 μg/ml) of cycloheximide primarily inhibit transporter synthesis, whereas high concentrations (50 μg/ml) of cycloheximide inhibit both transporter synthesis and inactivation or turnover.

Modulation of hexose transport rates in insulin-sensitive fat and muscle (2–7) cells often correlates with changes in the subcellular distribution of glucose transporter proteins. How-
ever, there is a growing body of evidence that suggests that the catalytic activity of glucose transporter proteins is also subject to regulatory control. Discrepancies between the magnitudes of insulin-induced increases in glucose transport rates (10–20-fold) and in the increased amounts of transporter proteins in the plasma membrane fraction (2–5-fold) from rat adipocytes suggest that changes in the intrinsic catalytic activity of glucose transporter proteins may also occur (37). Furthermore, other apparent dissociations between the extent of transporter redistribution and glucose transport rates have been reported. For example, agents that increase the activity of the cAMP-dependent protein kinase, such as isoproterenol, adrenocorticotropic hormone, glucagon, and dibutyryl cAMP, acutely inhibit insulin-stimulated glucose transport activity in isolated rat adipocytes (38–41). This effect on glucose transport is not due to detectable changes in the amounts of glucose transporter proteins in the plasma membrane fraction, as measured by [H]cytochalasin B binding, and may be due to a decrease in the intrinsic catalytic activity of glucose transporters (17). In contrast, it has recently been shown (17) that chronic exposure of 3T3-L1 adipocytes to cholera toxin or dibutyryl cAMP increases hexose transport. This effect appears due in part to an increase in the catalytic activity of one or both transporter isoforms present in these cells. In addition, treatment of 3T3-L1 fibroblasts and adipocytes with micromolar concentrations of cadmium dramatically increases hexose transport in both cell types without associated increases in the amounts of GLUT1 or GLUT4 protein in the plasma membrane fractions when compared with control cells.

The most striking findings reported here relate to the observed discrepancies between the fold increases in hexose transport rates and the relative amounts of the transporter isoforms in the plasma membrane fractions from cells treated with insulin, anisomycin, or cycloheximide. The action of insulin results in an ~30–50% decrease in the amounts of GLUT1 and GLUT4 proteins in the low density microsomal fraction (Fig. 5) (17, 42). This effect is associated with 1.6- and 2.8-fold increases in the relative amounts of GLUT1 and GLUT4 proteins, respectively, in the plasma membrane fraction (Fig. 6) (17, 42). These changes in the subcellular distributions of the glucose transporters are associated with a ~10-fold increase in hexose transport rates. This discrepancy between the magnitudes of the increases in plasma membrane glucose transporters and hexose transport rates suggests that insulin may increase the intrinsic catalytic activity of the glucose transporter proteins. Alternatively, it is possible that the plasma membrane fraction, as prepared in this study, is contaminated with membrane fractions containing intracellular glucose transporters. This would lead to an underestimation of the fold changes in cell-surface transporter amounts caused by the action of insulin. However, the concept that the intrinsic catalytic activity of the glucose transporter proteins can indeed be regulated is strongly reinforced by the results obtained when cells are treated with anisomycin or cycloheximide. Treatment of 3T3-L1 adipocytes with either protein synthesis inhibitor for 5 h does not significantly affect the subcellular distributions of any detectable glucose transport isoform (Figs. 5–7). Nevertheless, the actions of anisomycin and cycloheximide elicit ~5- and 5-fold increases, respectively, in hexose transport rates when compared to control cells (Figs. 1, 2, and 6). Taken together, these results strongly suggest that the amounts of GLUT1 and GLUT4 proteins at the surface of 3T3-L1 adipocytes are not sufficient to account for the increases in hexose transport rates elicited by the actions of anisomycin or cycloheximide. Thus, the cellular actions of these agents lead to marked increases in the intrinsic catalytic activity of one or more transporter isoforms in 3T3-L1 adipocytes. This conclusion differs from those of two earlier investigations (9, 10) of the effect of cycloheximide on the activity of glucose transporter proteins and their cellular distribution in isolated rat adipocytes. In these studies, treatment of rat adipocytes with cycloheximide increased the catalytic activity of glucose transporters only if these cells were subsequently treated with insulin. In contrast, Jones and Cushman (11) did not observe any changes in the catalytic activity of glucose transporters in rat adipocytes exposed to cycloheximide with or without insulin.

The mechanism for the apparent increase in catalytic activity of 3T3-L1 adipocyte glucose transporter proteins by anisomycin or cycloheximide is unclear. It is known that anisomycin and cycloheximide are structurally unrelated and inhibit protein synthesis by different mechanisms (43). Cycloheximide blocks peptide bond formation, whereas anisomycin inhibits association of the ribosomal subunits (26). The fact that both these agents increase glucose transport activity in 3T3-L1 adipocytes makes it unlikely that this effect is a nonspecific one, unrelated to the inhibition of protein synthesis. Interestingly, in addition to the regulation of glucose transport activity, it has recently been demonstrated (44) that insulin, cycloheximide, or anisomycin can induce the transcription of the c-fos gene in rat H4IE hepatoma cells. Moreover, both insulin (45, 46) and cycloheximide (45, 47) potently stimulate the activity of one or more S6 kinases in many tissues and cultured cell lines. At present, it is not known if the activation of S6 kinase by either insulin or cycloheximide is an intermediate step in the cellular signaling pathway leading to the increase in glucose transport activity in 3T3-L1 adipocytes. A simple hypothesis for the increase in hexose transport by anisomycin and cycloheximide is that these agents inhibit the synthesis of one or more short-lived proteins that, in the basal state, suppress the activity of glucose transporter proteins at the cell surface. Suppression of this transport activity by such a putative protein could either be a direct or indirect consequence of an associated enzymatic activity. Additional studies in this laboratory are in progress to test the validity of this hypothesis.

Acknowledgments—We thank Drs. Anna Pessino and Anthony Carruthers for helpful advice and discussions during the course of this study. We also thank Dr. Michael F. Shanahan for generously providing us with [3H]IAPS-IAP-7877.

REFERENCES

Anisomycin and Cycloheximide Increase Glucose Transport