Hexose Transport Stimulation and Membrane Redistribution of Glucose Transporter Isoforms in Response to Cholera Toxin, Dibutyryl Cyclic AMP, and Insulin in 3T3-L1 Adipocytes*

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Exposure of 3T3-L1 adipocytes to 100 ng/ml of cholera toxin or 1 mM dibutyryl cyclic AMP caused a marked stimulation of deoxyglucose transport. A maximal increase of 10- to 15-fold was observed after 12- to 24-h of exposure, while 100 nm insulin elicited an increase of similar magnitude within 30 min. A short term exposure (4 h) of cells to cholera toxin or dibutyryl cyclic AMP resulted in a 3- to 4-fold increase in deoxyglucose transport which was associated with significant redistribution of both the HepG2/erythrocyte (GLUT1) and muscle/adipocyte (GLUT4) glucose transporters from low density microsomes to the plasma membrane fraction. Total cellular amounts of both transporter proteins remained constant. In contrast, cells exposed to cholera toxin or dibutyryl cyclic AMP for 12 h exhibited elevations in total cellular contents of GLUT1 (but not GLUT4) protein to about 1.5- and 2.5-fold above controls, respectively. Although such treatments of cells with cholera tox (12 h) versus insulin (30 min) caused similar 10-fold enhancements of deoxyglucose transport, a striking discrepancy was observed with respect to the content of glucose transporter proteins in the plasma membrane fraction. While insulin elicited a 2.6-fold increase in the levels of GLUT4 protein in the plasma membrane fraction, cholera tox increased the amount of this transporter by only 30%. Insulin or cholera tox increased the levels of GLUT1 protein in the plasma membrane fraction equally (1.6-fold). Thus, a greater number of glucose transporters in the plasma membrane fraction is associated with transport stimulation by insulin compared to cholera tox.

We conclude that: 1) at early times (4 h) after the addition of cholera tox or dibutyryl cyclic AMP to 3T3-L1 adipocytes, redistribution of glucose transporters to the plasma membrane appears to contribute to elevated deoxyglucose uptake rates, and 2) the stimulation of hexose uptake after prolonged treatment (12-18 h) of cells with cholera tox may involve an additional increase in the intrinsic activity of one or both glucose transporter isoforms.

One of the most important physiological effects of insulin is its ability to increase glucose transport in cardiac and skeletal muscles and adipose tissue (1). Many metabolic pathways such as glycogen and fatty acid synthesis are influenced as a consequence of the increase in intracellular glucose and its metabolites. The insulin-stimulated increase in glucose transport in fat and muscle cells is associated with a redistribution of glucose transporter proteins from an intracellular pool to the plasma membrane (2-7). This effect is dependent upon cellular energy stores and is reversed by the removal of insulin from cells using collagenase (8) or anti-insulin antibodies (9). 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.

Recenty, a number of studies have demonstrated that several glucose transporter protein isoforms are present in mammalian cells. Immunological (10-12) and recombinant DNA technologies (13-16) have revealed the coexistence of at least two glucose transporter proteins, designated as GLUT1 and GLUT4,1 in insulin-sensitive rat adipocytes and rat skeletal muscle. Both GLUT1 and GLUT4 are redistributed from low density microsomes to the plasma membrane in rat adipocytes exposed to insulin (17). However, the magnitude of the increase in the amount of GLUT4 in plasma membranes is much greater than that observed for GLUT1 (17).

It is not clear if the action of insulin also modulates the intrinsic activity of glucose transporters. Discrepancies between the magnitudes of insulin-induced increases in glucose transport rates (10-20-fold) and in the amounts of transporter proteins in plasma membranes (2-5-fold) from rat adipocytes suggest that changes in the activity of glucose transport proteins may occur (18). 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, adrenocorticotrophic hormone, glucagon, and Bt2cAMP,2 acutely inhibit insulin-stimulated glucose transport activity in rat adipocytes (19-22). This effect on glucose transport is not due to detectable changes in the amounts of glucose transporters in the plasma membrane, as measured by [3H]cytochalasin B binding, and

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1 GLUT1 and GLUT4 are designations for the erythrocyte/HepG2 glucose transporter and the adult skeletal muscle/insulin-responsive glucose transporter, respectively, adopted from the nomenclature of Fukumoto et al. (15).

2 The abbreviations used are: Bt2cAMP, dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; [3H]IAPS, 3-[3H]iodo-4-azidophenethylamino-7-O-succinyl-deactyl.
may to be due to a decrease in the intrinsic activity of glucose transporters (22, 23). Although rat adipocytes possess two glucose transporters, GLUT4 appears to represent about 90–95% of the transporter complement in these cells (11, 17). Therefore, the inhibitory effect on glucose transport by agents which increase the activity of the cAMP-dependent protein kinase in these cells may reflect changes primarily in the activity of GLUT4.

The aim of the present work was to evaluate further whether glucose transporter activity changes accompany cAMP action by specifically monitoring both GLUT1 and GLUT4 in membrane fractions from insulin-responsive cells. Differentiated 3T3-L1 cells in culture were used as a model system in these studies. These cells respond to insulin with 10–15-fold increases in glucose transport, and, unlike suspensions of rat adipocytes, can be maintained in a viable, hormone-sensitive state for several days. Like rat adipocytes, differentiated 3T3-L1 cells express both GLUT1 (24) and GLUT4 (13) proteins, although the relative amounts of each isoform have not been quantitated. Surprisingly, we observed that differentiated 3T3-L1 cells exposed to cholera toxin or B2cAMP exhibit large increases in glucose transport. The time courses for this effect coincides with an increase in total cellular GLUT1 after 12-24 h of exposure. Notably, exposures of cells to cholera toxin for 12 h or insulin for 0.5 h are equally effective with respect to the stimulation of glucose transport and the increase in the amounts of GLUT1 in isolated plasma membranes. However, the marked redistribution of GLUT4 from low density microsomes to plasma membranes elicited by insulin is not observed in cells exposed to cholera toxin. These observations suggest that an apparent increase in the intrinsic activity of one or both transporter proteins may account for the effect of cholera toxin on glucose transport in differentiated 3T3-L1 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

2-Deoxy-D-glucose, cytochalasin B, bovine insulin (for inducing cell differentiation) were purchased from Sigma. Porcine insulin (for in vitro assays) was a gift from Dr. Ronald Chance, Lilly Research Laboratories. Cholera toxin, pertussis toxin, and the B subunit of cholera toxin were purchased from List Biological Laboratories, Inc. Monoclonal antibody 1F8 was a generous gift from Dr. Paul Pilch (Dept. of Biochemistry, Boston University School of Medicine, Boston, MA). 5'-[3H]Br-cAMP was a generous gift (from Dr. Michael F. Shannahon, Dept. of Physiology, Southern Illinois University School of Medicine, Carbondale, IL). 3T3-L1 fibroblasts were purchased from the American Type Culture Collection. DMEM, antibiotics, and fetal bovine serum were purchased from GIBCO. 2-Deoxy-D-[1,2-3H]glucose was purchased from Amersham Corp. [3H]-labeled Protein A (5-10 Ci/μg) and [3H]-cAMP were purchased from Du Pont-New England Nuclear.

**Methods**

**Cell Cultures—**The 3T3-L1 fibroblasts used in this study were seeded at a density of 50 cells/mm². The fibroblasts were grown in DMEM containing 10% calf serum, 5 units/ml of penicillin, and 50 μg/ml of streptomycin sulfate (28 ml of medium/100 × 20-mm plate or 1 ml of a 12-well culture plate) and maintained in a 5% CO₂ humidified atmosphere at 37 °C. This media was changed every 2–3 days. Several days after the cells achieved confluence, differentiation was induced with the same volumes of DMEM containing 10% fetal bovine serum and antibiotics, with media changes at 2–3-day intervals. Cells were used 10–14 days after the induction of differentiation at which time greater than 95% of the cells expressed the adipocyte phenotype.

**2-Deoxyglucose Glucose Transport—**Transport in differentiated 3T3-L1 cells was measured using a modification of the method described by Frost and Lane (25). Briefly, cells were grown in 12-well culture plates (Corning, containing approximately 8 × 10⁴ cells/well at the time of the assay), and incubated in 1 ml of DMEM containing 10% calf serum and antibiotics ± test agent as indicated. Before each assay, cells were incubated in 1 ml of serum-free DMEM ± test agent as indicated for 1.5 h at 37 °C. Next, the cells were washed twice with 1 ml of Krebs-Ringer phosphate buffer (130 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1.3 mM MgSO₄, 10 mM Na₂HPO₄, pH 7.4) and incubated in 1 ml of the same buffer ± test agent for 0.5 h. Glucose transport was determined in the absence or presence of 0.1 mM 2-deoxy-D-[2,6-3H]glucose (1 μCi) for 5 min at 37 °C. Transport was terminated by the rapid removal of assay buffer from each well followed by two rapid washes of each well with 1 ml of ice-cold Krebs-Ringer phosphate buffer. Cells were removed from each well with 0.4 ml of 0.1% SDS and counted after the addition of 4 ml of Optitfluor (Packard Instrument Co.). Nonspecific glucose transport was defined as that which occurred in the presence of 10 μM cytochalasin B. Under the conditions described, 2-deoxyglucose transport was linear from 1 to 30 min. The results of glucose transport assays performed in 150 × 20-mm culture plates (Nunc) were in good agreement with those performed in the 12-well culture plates.

**Cell Membrane Preparations—**Subcellular membranes from 3T3-L1 adipocytes were prepared using modifications of the methods described by Simpson and Paruszewski (26). Briefly, cells were grown in 20-mm culture plates (two plates/condition), and incubated in 38 ml of DMEM containing 10% fetal bovine serum and antibiotics ± test agent as indicated. Before homogenization the cells were incubated in the same volume of serum-free DMEM ± test agent for 2 h at 37 °C. Next, the cells were washed once with 10 ml of Buffer A (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) at 37 °C. Monolayers from two plates were scraped into 34 ml of ice-cold Buffer A and were immediately homogenized with 15 strokes of a motor-driven Teflon pestle in a 40-ml homogenization vessel (Wheaton).

Buffer A was used throughout the differential centrifugation protocol and all samples were kept at 4 °C. A 3-ml aliquot of the homogenate was subjected to centrifugation at 250,000 × g for 1 h to collect total cellular membranes which were resuspended to about 5 mg of protein/ml in Buffer B (20 mM HEPES, 1 mM EDTA, pH 7.4). The remainder of the homogenate was subjected to centrifugation at 100,000 × g for 20 min. The pellet was resuspended in 6 ml of Buffer B with a Dounce homogenizer, applied to a sucrose cushion (1.12 M sucrose in Buffer B), and subjected to centrifugation at 100,000 × 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 × g for 0.5 h. Plasma membranes were resuspended in Buffer B to about 5 mg of protein/ml. The material which collected beneath the sucrose cushion, containing nuclei, mitochondria, and broken cells, was also resuspended in Buffer B to about 5 mg of protein/ml.

The supernatant from the 16,000 × g centrifugation was subjected to centrifugation at 30,000 × g for 30 min. The resulting supernatant was then subjected to centrifugation at 250,000 × g for 1.5 h to collect the low density microsomes which were resuspended in Buffer B to about 5 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 Wallace (27). Briefly, 5'-nucleotidase was assayed in a 1-ml volume containing 50 μmol of Tris-Cl (pH 7.4), 10 μmol of MgCl₂, 0.675 μmol of AMP, a tracer amount of [3H]-AMP and membrane suspension. Samples were incubated at 37 °C for 20 min. The reaction was terminated by the addition of 0.25 M ZnSO₄. Protein and unhydrolyzed AMP were precipitated by the addition of 0.23 ml of 0.3 N Ba(OH)₂ and adenosine remained in solution. After centrifugation, a 0.4-ml aliquot of the supernatant was removed from each tube and counted in 4 ml of Optifluor (Packard). The specific activity (nanomoles/min/mg of protein) of 5'-nucleotidase in cellular homogenates, plasma membranes, low density microsomes, nuclei/mitochondria fraction, and plasma membranes was 5, 12, 4, 5, and 46, respectively, for control cells. As has been shown previously for insulin (28), exposure of 3T3-L1 adipocytes to cholera toxin or B2cAMP did not change this 9-fold enrichment in 5'-nucleotidase-specific activity in plasma membranes.
Production of GLUT1 Antisera—A peptide, SDKPPEELFPHLGADSVQ, corresponding to amino acids 475-492 of human GLUT1 (29), was synthesized on an Applied Biosystems Model 430A solid-phase peptide synthesizer (University of Massachusetts Medical Center Peptide Synthesis Facility). The peptide was purified by reverse-phase high pressure liquid chromatography in 0.1% trifluoroacetic acid with a linear 40-75% gradient of acetonitrile through a preparative C4 column (Vydac). The purified peptide was coupled to rabbit serum albumin with glutaraldehyde (30). Rabbits were immunized (East Acres Biologicals, Southbridge MA) with the peptide-albumin conjugate, as described by Oka et al. (11).

Analysis of Glucose Transport Proteins in Subcellular Membranes—For each experiment, aliquots of subcellular membrane fractions were subjected to SDS-polyacrylamide (10%) gel electrophoresis, in duplicate gels, under reducing conditions as described by Laemmli (31). Resolved proteins were then electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell, pore size of 0.45 μm) at 200 mA for 2 h in transfer buffer (150 mM glycine, 20 mM Tris, 20% methanol, pH 8.2). Duplicate nitrocellulose filters were then immersed in blocking 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 used for subsequent washes and as the solvent for the antibody and Protein A solutions was composed of 250 mM NaCl, 20 mM Tris, 0.1% Tween-20, and 0.05% Tween-20 (pH 7.5). One of the duplicate nitrocellulose filters was incubated in a solution of R-480 antisera (anti-C-terminal GLUT1 peptide), diluted 1:1000 for 12 h at 4°C. The other duplicate filter was incubated in a solution of monoclonal antibody IF8 (anti-GLUT4), affinity purified on immobilized Protein A, and diluted 1:250 for 12 h at 4°C. After exposure to the primary antibody solutions, nitrocellulose filters were washed six times for 4 min at room temperature. Papers previously exposed to the IF8 antibody solution were then incubated in a solution of rabbit anti-mouse IgG (Cappel, total IgG at 4 mg/ml), diluted 1:2000 for 45 min at room temperature, and washed as described above.

To detect immunoreactivity, nitrocellulose filters were incubated in a solution of 125I-labeled Protein A at 0.5 μCi/ml for 45 min at room temperature. Dried filters were subjected to autoradiography (Kodak X-Omat film) at −70°C with a DuPont Lightening Plus intensifying screen. Immunoreactivity was quantitated by cutting out autoradiographs or GLUT1 or GLUT4 on the developed films, from the nitrocellulose filters and present on a size-matched strip of the filter cut from an area above or below each immunoreactive transporter band.

RESULTS

Effects of Insulin, Cholera Toxin, and Bt2cAMP on 2-Deoxyglucose Transport—As expected, the addition of 100 nM insulin to 3T3-L1 adipocytes elicited a rapid (within 30 min) 10-15-fold increase in 2-deoxyglucose uptake over that observed in control cells (Fig. 1). Exposure of cells to 100 ng/ml of cholera toxin or 1 mM Bt2cAMP also increased 2-deoxyglucose uptake. However, in contrast to insulin action, the stimulatory effects of cholera toxin and Bt2cAMP were observed between 12 and 24 h, there were differences in the time courses of these effects. Cells exposed to cholera toxin exhibited a sigmoidal increase in deoxyglucose uptake, whereas the effect elicited by Bt2cAMP was biphasic with an apparent plateau between 2 and 4 h of exposure (Fig. 1).

The cellular uptake of 2-deoxyglucose is the biochemical result of two tightly coupled events, i.e. substrate transport and its phosphorylation by hexokinase. Therefore, in other experiments (not illustrated), 3-O-methylglucose transport in 3T3-L1 adipocytes was measured under conditions similar to those described in Fig. 1. This hexose is specifically transported but not phosphorylated in mammalian cells. Cells exposed to 100 nM insulin or 100 ng/ml of cholera toxin for 0.5 and 24 h, respectively, showed similar increases in 3-O-methylglucose transport, measured at 30 s, compared to control cells. Therefore, under the conditions described in this study, the stimulatory effects of cholera toxin and Bt2cAMP on 2-deoxyglucose uptake in differentiated 3T3-L1 cells reflect increases in the transport systems for this sugar.

The effect of the cholera toxin B subunit, necessary for holotoxin binding to the cell surface, was examined to determine if the effect of cholera toxin was simply due to its binding to the cell surface ganglioside GM1. As shown in Fig. 2, an 18-h exposure of cells to 100 ng/ml of the B subunit only slightly increased deoxyglucose transport compared to the effect of insulin, suggesting that the full stimulatory effect of cholera toxin is dependent upon the presence and activity of the catalytic (A) subunit. An 18-h exposure of 3T3-L1 cells to 100 ng/ml of pertussis toxin had no significant effect on deoxyglucose transport. Insulin was able to fully activate deoxyglucose transport in cells previously exposed to either pertussis toxin or the B subunit of cholera toxin, suggesting that these agents do not adversely affect cell viability or function with respect to glucose transport.

Effects of Insulin, Cholera Toxin, and Bt2cAMP on Cellular Amounts of Glucose Transport Proteins—After appropriate treatment, differentiated 3T3-L1 cells were homogenized and subcellular membrane fractions were isolated to examine the effects of 100 nM insulin, 100 ng/ml of cholera toxin, or 1 mM Bt2cAMP on the cellular amounts of GLUT1 and GLUT4.

3 B. M. Clancy and M. P. Czech, unpublished observations.
None of the agents tested at various times of exposure affected the amounts of total protein recovered in subcellular membrane fractions. The yields of total cellular membranes, plasma membranes, and low density microsomes from 1 150 × 20-mm culture plate were approximately 6, 0.6, and 0.5 mg of protein, respectively. Immunoblots of the time-dependent effects of insulin, cholera toxin, and Bt,cAMP on the amounts of GLUT1 and GLUT4 in total cellular membranes are shown in a representative experiment (Fig. 3) in which the effects of cholera toxin and Bt,cAMP were qualitatively similar. Western blots from several independent experiments were quantitated as shown in Fig. 4. In cells exposed to either cholera toxin or Bt,cAMP there was a significant, time-dependent increase in the amount of GLUT1 which was maximal between 12 and 24 h. This effect of Bt,cAMP on the amount of GLUT1 in total cellular membranes was almost two times greater than that elicited by cholera toxin. As suggested by the autoradiograph in Fig. 3, exposure of cells to Bt,cAMP had no significant effect on the amount of GLUT4 in total cellular membranes. Cholera toxin also did not alter the amount of GLUT4 for as long as 18 h of exposure, but consistently decreased the amount of GLUT4 when cells were exposed to this reagent for 24 h (Fig. 4). Addition of 100 nM insulin to 3T3-L1 adipocytes had divergent effects on the total cellular amounts of GLUT1 and GLUT4. In cells exposed to insulin for 2 and 24 h the relative amounts of GLUT1 were 1.12 ± 0.12 (n = 5) and 1.64 ± 0.30 (n = 3), respectively; and the relative amounts of GLUT4 were 1.10 ± 0.10 (n = 3) and 0.57 ± 0.01 (n = 3), respectively. Thus, prolonged exposure of differentiated 3T3-L1 cells to 100 nM insulin or 100 ng/ml of cholera toxin results in a significant decrease in total cellular GLUT4.

Effects of Insulin, Cholera Toxin, and Bt,cAMP on Membrane Distributions of Glucose Transport Proteins—To understand if the stimulatory effects of 100 ng/ml of cholera toxin and 1 mM Bt,cAMP on deoxyglucose transport were related to changes in the distributions of glucose transporters, the relative amounts of GLUT1 and GLUT4 in subcellular membrane preparations were examined. The distributions of GLUT1 in plasma membranes and low density microsomes from cells exposed to cholera toxin or Bt,cAMP for 4 and 18 h were quantitated and compared to those from control and insulin-treated cells (Fig. 5). A 4-h exposure of cells to cholera toxin or Bt,cAMP consistently decreased the amount of GLUT1 in low density microsomes. This effect (20–30% decrease) was much less pronounced than that elicited by insulin (60% decrease). Insulin increased the amount of GLUT1 in plasma membranes by 25% in this series of experiments. A 4-h exposure of cells to cholera toxin or Bt,cAMP also increased the amount of GLUT4 in plasma membranes, but less than that elicited by insulin. In cells exposed to cholera toxin or Bt,cAMP for 18 h, the amounts of GLUT1 in both plasma membranes and low density microsomes were increased com-
Fig. 4. Analysis of the time-dependent effects of cholera toxin (CTx) and Bt2cAMP on the relative amounts of GLUT1 and GLUT4 proteins in total membranes from 3T3-L1 adipocytes. Western blots from several experiments like the one shown in Fig. 3 (SDS-polyacrylamide gel electrophoresis with 100-200 mg of total membrane proteins) were quantitated as described under “Experimental Procedures.” Specific binding of 125I-labeled Protein A to glucose transporters on immunoblots was determined and expressed in units relative to time 0 (control). The results presented are the means and standard errors from at least four and three experiments for GLUT1 (○) and GLUT4 (□), respectively. Not shown are the results obtained with 100 nM insulin: GLUT1 at 2 and 24 h, 1.12 ± 0.12 (n = 5) and 1.64 ± 0.30 (n = 3), respectively; GLUT4 at 2 and 24 h, 1.10 ± 0.10 (n = 3) and 0.57 ± 0.01 (n = 3), respectively. Note the different scales for the ordinates in panels A and B. Asterisk, in each experiment the values of the relative immunoreactivity were always greater than control. Double asterisks, in each experiment the values of the relative immunoreactivity were always less than control.

The distributions of GLUT4 in 3T3-L1 cell membrane fractions are shown in Fig. 6. Compared to control cells, there was a 60% decrease in the relative amount of GLUT4 in low density microsomes, when compared to values observed at 4 h.

The relative amounts of GLUT4 in plasma membranes from cells exposed to cholera toxin and Bt2cAMP were determined and expressed in units relative to the control (C). The bar graph shows the means and ranges from two experiments. Above the bar graph are the relevant strips of a representative Western blot.
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FIG. 6. The distributions of GLUT4 protein in plasma membranes (PM) and low density microsomes (LDM) from 3T3-L1 adipocytes. See legend of Fig. 5 for details. Protein immunoblot analysis of nitrocellulose filters was performed with monoclonal antibody 1F8 (anti-GLUT4). Specific binding of 3H-labeled Protein A to GLUT4 protein was determined and expressed in units relative to the control (C). The bar graph shows the means and ranges from two experiments. Above the bar graph are the relevant strips of a representative Western blot.

to those obtained in cells exposed to insulin for 0.5 h. The effects of cholera toxin and Bt2cAMP, in the presence or absence of insulin, on the amounts of GLUT1 and GLUT4 in plasma membranes are shown in a representative immunoblot (Fig. 7). The results from several similar experiments were also quantitated by direct measurement of radioactivity in the transporter bands, as shown in Fig. 8, panel A. As expected, insulin elicited an increase in the amounts of both transporter proteins in the plasma membrane fraction. The insulin-induced increase in the amount of GLUT4 (2.5-fold) was greater than that for GLUT1 (1.65-fold), consistent with the data in Figs. 5 and 6. The amount of GLUT1 in plasma membranes from cells exposed to cholera toxin or Bt2cAMP increased to levels equal to or greater than that observed for insulin. The amounts of GLUT4 in this fraction were affected much less by these agents compared to the action of insulin. The relative increases in the amount of GLUT1 in plasma membranes from cells exposed to cholera toxin or Bt2cAMP were consistent with the relative increases in the total cellular amount of GLUT1 at 12 h as shown in Fig. 4.

FIG. 7. The effects of insulin, cholera toxin (CTx); and Bt2cAMP on the amounts of GLUT1 and GLUT4 proteins in plasma membranes from 3T3-L1 adipocytes. Cells in 150 x 20-mm culture plates were exposed to 100 ng/ml of cholera toxin (CTx) or 1 mM Bt2cAMP at 37 °C for the times indicated. Where indicated, cells were also exposed to 100 nM insulin (I) for 0.5 h at 37 °C before cells were homogenized. Plasma membranes were prepared as described under “Experimental Procedures.” Plasma membrane proteins (100 mg) were resolved with SDS-polyacrylamide (10%) gel electrophoresis in duplicate gels, transferred to nitrocellulose filters, and then protein immunoblot analyses were performed with either R-480 antisera (anti-GLUT1) or monoclonal antibody 1F8 (anti-GLUT4).

In order to assess the functional consequences of the effects of insulin, cholera toxin, and Bt2cAMP on the cellular distributions of GLUT1 and GLUT4 (Fig. 8, panel A), deoxyglucose transport was measured under the same conditions (Fig. 8, panel B). A striking finding revealed by this comparison was the discrepancy between the distributions of both glucose transporters in plasma membranes and the deoxyglucose transport rates in cells exposed to insulin for 0.5 h as opposed to either cholera toxin or Bt2cAMP for 12 h. In particular, in cells exposed to insulin the increase in the relative amounts of GLUT4 was much greater than the increase in the relative amounts of GLUT1 in plasma membranes. In contrast, in cells exposed to either cholera toxin or Bt2cAMP the increase in the relative amounts of GLUT1 was greater than the increase in the relative amounts of GLUT4 in plasma membranes.

Assuming unaltered transporter intrinsic activity, the increases in the amounts of GLUT1 and GLUT4 in plasma membranes do not appear sufficient to account for the markedly activated rates of hexose transport in response to any of these stimulants, especially cholera toxin. Cholera toxin or insulin alone similarly stimulated deoxyglucose transport about 13-fold over control. These agents also elicited about a
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Plasma Membranes

A. Immunoreactivity (Relative units)

- GLUT 1
- GLUT 4

B. 2-Deoxyglucose Transport (Relative units)

- I - + - + - + 0.5 h
- CTx - + - + - + 12 h
- Bt-cAMP - + - + - + 0.5 h

Fig. 8. A comparison between the effects of insulin, cholera toxin (CTx), and Bt-cAMP on the relative amounts of GLUT1 and GLUT4 in plasma membranes and the relative increases in 2-deoxyglucose transport in 3T3-L1 adipocytes. Cells were exposed to 100 ng/ml of cholera toxin or 1 mM Bt-cAMP for 12 h at 37°C. Where indicated, cells were also exposed to 100 nM insulin (I) for 0.5 h at 37°C before the experiment was performed. A, Western blots from several experiments like the one shown in Fig. 7 (SDS-polyacrylamide gel electrophoresis with 75–150 mg of plasma membrane proteins) were quantitated as described under “Experimental Procedures.” Specific binding of 125I-labeled Protein A to glucose transporters was determined and expressed in units relative to control. The results presented are the means and standard errors from four (Bt-cAMP ± I) and five (CTx ± I) experiments. B, 2-deoxyglucose transport in 12-well culture plates was measured as described under “Experimental Procedures.” The results presented are the means and standard errors from four experiments.

1.6-fold increase in the amount of GLUT1 in plasma membranes. However, only half as much GLUT4 was present in plasma membranes derived from cholera toxin versus insulin-treated cells (Fig. 8, panel A). Furthermore, exposure of cells to the combination of insulin and cholera toxin almost doubled deoxyglucose transport rates compared to either agent alone, yet the amounts of GLUT1 and GLUT4 in plasma membranes were essentially unchanged compared to insulin treatment alone.

Similarly, Bt-cAMP elicited a 13-fold increase in deoxyglucose transport in 3T3-L1 adipocytes (Fig. 8, panel B). However, the distributions of GLUT1 and GLUT4 in plasma membranes were different compared to the effects of cholera toxin and insulin. In cells exposed to Bt-cAMP for 12 h the relative increases in GLUT1 and GLUT4 were about 2.5- and 1.8-fold, respectively. Exposure to both insulin and Bt-cAMP further increased deoxyglucose transport rates compared to either agent alone. This effect was associated with small increases in the amounts of GLUT1 and GLUT4 in the plasma membrane fraction.

Fig. 9. The effects of insulin, cholera toxin (CTx), and Bt-cAMP on the amounts of GLUT1 protein in low density microsomes from 3T3-L1 adipocytes. See legend of Fig. 7 for details. Low density microsomal proteins (50 mg) were resolved with SDS-polyacrylamide (10%) gel electrophoresis, transferred to nitrocellulose filters, and then protein immunoblot analysis was performed with R-480 antisera (anti-GLUT1).

The experiments depicted in Figs. 5 and 6 showed that a short exposure of cells to either cholera toxin or Bt-cAMP consistently decreased the amounts of GLUT1 and GLUT4 in low density microsomes. We next tested whether the similar effect of insulin to decrease the amounts of these transporters in low density microsomes was additive to that of cholera toxin and Bt-cAMP. As shown in Fig. 9, exposing cells to any of these agents alone decreased the amount of GLUT1 in low density microsomes and insulin was the most effective in this respect. Importantly, the combination of insulin and either cholera toxin or Bt-cAMP decreased the amount of GLUT1 in low density microsomes more than any of these agents acting alone. Similar results were obtained when the amounts of GLUT4 were measured in low density microsomes (not shown). These results indicate that intracellular low density microsomal membranes containing GLUT1 and GLUT4 retained their full ability to respond to the insulin stimulus even after their partial depletion of glucose transporters by cholera toxin or Bt-cAMP.

DISCUSSION

The results presented in this paper demonstrate a marked stimulatory effect of 100 ng/ml of cholera toxin and 1 mM Bt-cAMP on hexose transport in differentiated 3T3-L1 cells in culture. Similar data are obtained when either 2-deoxyglucose (Fig. 1) or 3-O-methylglucose transport (not illustrated) rates are measured. The extent of the maximal increase in deoxyglucose transport rates in cells exposed to either cholera toxin or Bt-cAMP is 10–15-fold, equivalent to that observed in cells exposed to 100 nM insulin (Figs. 1 and 8). However, the onset of the increase in deoxyglucose transport elicited by cholera toxin and Bt-cAMP is much slower than that elicited by insulin. While the insulin effect is essentially maximal after 30 min of incubation, deoxyglucose transport rates in cells exposed to cholera toxin or Bt-cAMP approach those observed for insulin only after exposures of 12–24 h (Fig. 1). It is also apparent that there are differences in the time courses for the increase in deoxyglucose transport elicited by cholera toxin and Bt-cAMP. The time course for the increase in deoxyglucose transport in cells exposed to Bt-cAMP is biphasic, associated with a distinct plateau in the effect between 2 and 4 h after the addition of this reagent. However, the time course for the increase in deoxyglucose transport induced by cholera toxin is sigmoidal, characterized by a short
lag time between the addition of this reagent and the onset of this effect. Similar time courses of action have also been observed for cholera toxin-induced increases in the amounts of cAMP and lipolysis in rat adipoma 3T3 cells (32) and isolated rat adipocytes (33), respectively. In addition to cholera toxin and Bt-cAMP, exposure of differentiated 3T3-L1 cells to 10 μM isoproterenol for 1 h or 20 μM forskolin for 24 h elicited about 4- and 8-fold increases, respectively, in deoxyglucose transport under the conditions of our experiments.5

The results in this study are consistent with earlier reports on the effects of agents which increase the amounts of cAMP or mimic the actions of cAMP in glucose transport in various cell types. Cholera toxin, Bt-cAMP, and 8-bromo-cAMP significantly increase glucose transport in primary cultures of dog thyroid cells (34). In addition, thyroid stimulating hormone has been reported to increase glucose transport in cultured rat thyroid cells (FRTL-5 cells) via elevated cAMP levels (35). More recently, Hiraki et al. (36) showed that cholera toxin and 8-bromo-cAMP significantly increase deoxyglucose uptake in NIH 3T3 fibroblasts after 6-h exposures to these reagents. Importantly, the B subunit of cholera toxin and pertussis toxin are not able to markedly increase deoxyglucose transport (Fig. 2) in differentiated 3T3-L1 cells. Taken together, the data from this study and earlier reports suggest that an undefined cellular signalling pathway, mediated by increased cellular cAMP concentrations and increased activity of the cAMP-dependent protein kinase, may regulate glucose transport in these cell types.

The results presented here with differentiated 3T3-L1 cells contrast with those obtained in analogous investigations of glucose transport in isolated rat adipocytes. A number of studies have demonstrated that agents such as isoproterenol or Bt-cAMP, which increase the cellular activity of cAMP-dependent protein kinase, acutely inhibit basal and insulin-stimulated glucose transport activity in primary rat adipocytes (19-22). The inhibitory effects of these agents on glucose transport in isolated rat adipocytes occur without accompanying changes in the amounts of glucose transporter proteins in the plasma membrane. Thus, these effects have been attributed to decreases in the intrinsic activity of cell-surface glucose transporters (19, 23). It is notable that direct G-protein involvement rather than a cAMP-dependent protein kinase pathway has been implicated in these actions of isoproterenol (19, 23). Presently, it is not clear why the effects of cholera toxin, Bt-cAMP, and isoproterenol on glucose transport differ so dramatically in differentiated 3T3-L1 cells and isolated rat adipocytes. Differentiated 3T3-L1 cells are morphologically similar to rat adipocytes and share a number of biochemical similarities (37). For example, in both cell types triglyceride synthesis is enhanced by insulin and inhibited by epinephrine (38). It remains to be seen if the differences in transport regulation in these cell types reflect differences in the relative amounts of GLUT1 and GLUT4.

Recent studies have demonstrated that the amounts and/or distributions of GLUT1 and GLUT4 are differentially regulated by agents which modulate glucose transport in the intact cell, e.g. insulin (17), streptozotocin (39, 40), and concentrations of glucose (41). Therefore, the prolonged time course of cholera toxin and Bt-cAMP action on glucose transport in 3T3-L1 adipocytes suggested that changes in transcription or protein translation may be involved. Experiments designed to determine whether protein synthesis is required for the effects of cholera toxin and Bt-cAMP on 3T3-L1 cells, at 12-24 hours of exposure, were not successful due to the fact that protein synthesis inhibition with 10 μg/ml of cycloheximide also stimulated hexose transport in these cells (not illustrated). However, both cholera toxin and Bt-cAMP increase the total cellular amounts of GLUT1 protein (Fig. 4). The magnitude of this response is greater with Bt-cAMP than cholera toxin in all experiments. The increase in the relative amount of GLUT1 may be due either to increased synthesis of this protein or to a decrease in its turnover. It should be noted that agents which increase intracellular cAMP, such as cholera toxin, forskolin, and 8-bromo-cAMP, significantly increase levels of mRNA for GLUT1 in NIH 3T3 fibroblasts within 3 h of exposure (36). Interestingly, the time courses of GLUT1 protein elevation by cholera toxin and Bt-cAMP parallel their effects on deoxyglucose uptake (Figs. 1 and 4). Therefore, it appears that the selective increase in the amount of GLUT1 in differentiated 3T3-L1 cells exposed to cholera toxin or Bt-cAMP may contribute to the changes observed in hexose transport rates.

In contrast to the changes in the amounts of GLUT1 described above, cells exposed to cholera toxin or Bt-cAMP do not exhibit marked changes in total cellular amounts of GLUT4. However, after 24 h of exposure to cholera toxin the amount of GLUT4 is consistently lower than control. Interestingly, exposure of 3T3-L1 cells to insulin for 24 h has opposite effects on the total cellular amounts of GLUT1 and GLUT4. After 24 h the amount of GLUT1 increases in response to insulin, while the amount of GLUT4 in total cellular membranes markedly decreases (Figs. 3 and 4). It is not known if the mechanism for this effect of insulin on the amount of GLUT4 is the same as that for cholera toxin. However, it is possible that increased turnover of GLUT4 occurs when it is present in the plasma membrane at high concentrations, as a result of insulin-induced redistribution. Recently, Tordjman et al. (42) reported that in differentiated 3T3-L1 cells exposed to 100 nM insulin for 72 h there were increases in deoxyglucose uptake, GLUT1 protein, and GLUT1 mRNA levels. In contrast, exposure of these cells to 100 nM insulin for 72 h did not significantly affect the levels of GLUT4 protein or mRNA.

In order to gain insight into the mode by which cholera toxin and Bt-cAMP increase glucose transport activity in 3T3-L1 cells, experiments were designed to measure subcellular membrane distributions of GLUT1 and GLUT4 proteins. These studies revealed several interesting observations. As previously reported for primary rat adipocytes (17), GLUT1 and GLUT4 proteins exhibit disparate distribution ratios between the plasma membrane and low density microsomal fractions of 3T3-L1 cells. These ratios are calculated to be approximately 1:1 and 1:5 (plasma membrane:low density microsomes) for GLUT1 and GLUT4, respectively, when cellular equivalents of plasma membrane and low density microsomes from 3T3-L1 cells are compared (Figs. 5 and 6). The relatively high amount of GLUT1 in the plasma membrane under basal conditions is associated with a relatively small increase due to insulin action compared to the GLUT4 protein (1.7-fold versus 2.5-fold, respectively). The decreases in the amounts of both GLUT1 and GLUT4 in low density microsomes from differentiated 3T3-L1 cells are about 50-60% (Figs. 5 and 6), indicating that these two transporters are similarly sensitive to the effect of insulin with respect to their redistribution from low density microsomes to the plasma membrane fraction. This observation is consistent with recent data from this and another laboratory (24) demonstrating the insulin-induced redistribution of human GLUT1 from low density microsomes to the plasma membrane fraction in 3T3-L1 cells transfected with human GLUT1.

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GLUT1 cDNA. Thus, the designation of GLUT4 as the insulin-regulatable glucose transporter isoform appears in appropriate with respect to its insulin-induced redistribution.

An important finding in the present studies is the significant decrease in low density microsomal content of both GLUT1 and GLUT4 following 4-h exposures of cells to cholera toxin or Bt₂CAMP (Figs. 5 and 6). These effects are accompanied by small (10-30%) increases in the amounts of these transporters in the plasma membrane fraction. Deoxyglucose transport is elevated about 5-6-fold at these early times of cell treatment with these agents (Fig. 1). These data indicate that redistribution of GLUT1 and GLUT4 proteins to the cell surface membrane may contribute to transport stimulation by cholera toxin and Bt₂CAMP at this time. However, the large disparity between the extent of transporter recruitment to the plasma membrane fraction and the extent of transport stimulation suggests that other processes may participate in the latter effect. Similar discrepancies between transporter redistribution and activity have been reported for insulin-stimulated glucose transport in rat adipocytes (18, 43, 44), suggesting that changes in transporter intrinsic activity by insulin plays a major regulatory role. In the present work, insulin elicits a 1.7- and 2.5-fold increase in the amounts of GLUT1 and GLUT4, respectively, in the plasma membrane fraction and this is associated with a 14-fold increase in deoxyglucose uptake (Fig. 8). This result is consistent with a concommitant change in transporter catalytic activity. However, we cannot unequivocally discount the possibility that the plasma membrane fractions obtained in these and other studies are contaminated with membrane fractions containing intracellular glucose transporters, leading to an underestimation of the changes in cell-surface transporter concentrations caused by insulin, cholera toxin, and Bt₂CAMP.

Due to the selectivity of our antibodies, these data also cannot exclude the possibility that another glucose transporter isoform may be present in 3T3-L1 adipocytes and may contribute to glucose uptake. If so, it is possible that exposure of these cells to cholera toxin or Bt₂CAMP significantly increases the amount of this undefined transporter in the plasma membrane. To examine this possibility we photolabeled glucose transporters present in plasma membrane fractions from control cells and cells exposed to 100 nM insulin (0.5 h), 100 ng/ml cholera toxin (12 h), or 1 mM Bt₂CAMP (12 h) with [³²P]IAPS-forskolin and then subjected the membrane proteins to SDS-polyacrylamide gel electrophoresis. It is assumed that IAPS-forskolin binds to GLUT1, GLUT4, and other glucose transporter isoforms with similar affinities and that incorporation of the photolabel occurs with the same or similar efficiencies for each protein. Total labeling of glucose transporters by this reagent was less intense in membranes from cells exposed to cholera toxin compared to those from insulin-treated cells, consistent with Fig. 9. This data does not support the hypothesis that a third glucose transporter isoform is responsible for the hexose transport effects induced by cholera toxin and Bt₂CAMP in 3T3-L1 adipocytes. However, these results do not disprove this possibility and other experiments are required to answer this question unequivocally.

Although the degree to which transporter recruitment to the plasma membrane in response to cholera toxin and Bt₂CAMP accounts for the enhanced hexose transport activity cannot be determined quantitatively, the effects of these agents on transporter distribution are almost additive to that of insulin (Figs. 7-9). Additional and rapid depletion of GLUT1 (Fig. 9) and GLUT4 (not illustrated) in low density microsomes, from cells previously exposed to cholera toxin or Bt₂CAMP, occurs in response to insulin. Similarly, plasma membrane levels of GLUT1 are higher in cells treated with insulin plus cholera toxin than either agent alone (Figs. 7 and 8). Interestingly, plasma membrane content of GLUT4 is maximal in response to insulin alone and increases no further in cells that were previously exposed to cholera toxin or Bt₂CAMP (Figs. 7 and 8). The reason for this is unclear at present. Nonetheless, the data in Fig. 8 suggest the possibility that different intracellular membrane populations containing glucose transporters are responsive to insulin versus cholera toxin or Bt₂CAMP. Further work will be required to test this hypothesis rigorously.

The most striking observations reported here relate to the apparent discrepancies between the effects of insulin versus cholera toxin on the fold increases in 2-deoxyglucose transport and the relative amounts of the two transporter isoforms in plasma membranes (Fig. 8). The marked glucose transport activity change in 3T3-L1 adipocytes due to cholera toxin treatment for 12 h is similar to that mediated by insulin. However, a large deficit in plasma membrane GLUT4 content is observed in cholera toxin-treated cells compared to those exposed to insulin. Plasma membrane GLUT1 levels are similar under these two conditions. Thus, the increase in hexose transport activity caused by cholera toxin is not accounted for by increased plasma membrane glucose transporters and appears to be associated with changes in transporter catalytic activity. This conclusion is reinforced by the results obtained when insulin plus cholera toxin are added to 3T3-L1 cells. Deoxyglucose transport is increased 25-fold over basal activity under these conditions. However, the amounts of GLUT1 and GLUT4 in plasma membranes remain at the levels observed in cells treated with insulin alone where only a 14-fold increase in transport activity is observed (Fig. 8). Taken together, these results indicate that the amounts of GLUT1 and GLUT4 at the 3T3-L1 cell surface are not sufficient to account for the exceedingly high hexose transport activity in the presence of cholera toxin unless the catalytic activities of one or both of these transporters are elevated compared to control cells.

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