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Biochemistry

GLUT4 facilitates insulin stimulation and cAMP-mediated inhibition of glucose transport

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ABSTRACT **The glucose transporter isoform GLUT4 is found only in cells that exhibit insulin-sensitive glucose transport. To investigate the function of this transporter, L6 myoblasts were stably transfected with GLUT4 cDNA. GLUT4 underwent insulin-dependent movement to the cell surface in myoblasts overexpressing the transporter. One cell line (243-6) expressed sufficient levels of the GLUT4 protein to study insulin-dependent glucose transport. Unlike wild-type L6 cells, 243-6 myoblasts exhibited two features that are characteristic of differentiated muscle fibers and adipocytes *in vivo*: a large insulin-stimulated component of glucose transport and inhibition of this stimulated component by cAMP. Relative to normal L6 cells, 243-6 cells responded to insulin or insulin-like growth factor 1 with a 5-fold larger increase in 2-deoxy[^{3}H]glucose uptake. $N^6,O^{2'}$-Dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) did not inhibit transport in normal L6 myoblasts, which express only GLUT1, but inhibited IGF-1/insulin-stimulated transport by 50% in 243-6 cells. The effect of cAMP was investigated further by using Chinese hamster ovary cells transiently expressing GLUT1 and GLUT4. Bt_2cAMP inhibited glucose transport only in Chinese hamster ovary cells expressing GLUT4. These results indicate that cAMP-mediated inhibition of glucose transport is dependent on expression of the GLUT4 isozyme.**

Insulin causes a marked and rapid increase in glucose transport in adipocytes, skeletal muscle cells, and cardiac myocytes (1, 2). This acute effect of insulin involves translocation of glucose transporters from an intracellular store to the plasma membrane (3, 4). β-Adrenergic agonists, such as epinephrine and isoproterenol, inhibit insulin-stimulated glucose transport (5–7). These inhibitory effects can be observed under conditions in which the level of plasma membrane transporters is not decreased (8, 9). Glucose transport is also decreased by cAMP derivatives (5, 6), suggesting that the inhibitory effects of β-adrenergic agonists are mediated by increased cAMP.

GLUT4 is one of five homologous glucose transport proteins (for review, see refs. 10 and 11). GLUT4 is expressed exclusively in those cell types exhibiting the highest levels of insulin-stimulated glucose transport (12, 13), although these cell types also express smaller amounts of GLUT1, a transporter isoform that has a much wider tissue distribution (14). GLUT1 is found in relatively high levels in the plasma membrane, where almost no GLUT4 can be detected under basal conditions (15–17). Insulin promotes translocation of both transporters to the cell surface (15–17); however, the magnitude of the insulin-dependent increase at the plasma membrane is much greater for GLUT4 than for GLUT1. Thus, GLUT4 appears to be primarily responsible for mediating insulin-stimulated transport in muscle and fat cells.

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L6 is a skeletal muscle cell line that has been previously used to investigate the regulation of glucose transport (18–20). Insulin stimulates glucose transport in L6 cells, but the magnitude of the stimulatory effect (2-fold maximum) is much less than that observed in rat skeletal muscles. Unlike skeletal muscle fibers *in vivo*, L6 myoblasts express very low levels of GLUT4 (21). The results described in the present report indicate that expression of GLUT4 *per se* can be sufficient to increase insulin-stimulated glucose transport in L6 myoblasts. In addition, evidence that expression of GLUT4 in L6 myoblasts and Chinese hamster ovary (CHO) cells results in the appearance of cAMP-dependent inhibition of glucose transport is presented.

MATERIALS AND METHODS

Cell Culture. L6 cells were cultured essentially as described (22). The cells (obtained from Gerald Fischbach, Harvard University) were subcloned and frozen. After thawing, cells were carried as myoblasts by culture in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. The growth medium was replaced every other day. To passage the cells, cultures were rinsed with solution A (135 mM NaCl/5.4 mM KCl/10 mM sodium phosphate, pH 7.4) and then incubated at 36°C in the same buffer containing 0.02% trypsin for 5 min. The cells were harvested by centrifugation and seeded into plastic culture dishes at a density of 3000 cells per cm^2. The number of passages was limited to 10.

CHO-K1 cells obtained from the American Type Culture Center (American Type Culture Collection) were cultured in DMEM containing 10% fetal calf serum. Baby hamster kidney cells (BHK-21, ATCC clone 13) were cultured in DMEM containing 7% fetal calf serum.

Transfection and Selection of Cells Expressing GLUT4. cDNA encoding rat GLUT4 (13) or a GLUT4 cDNA having a Ser → Ala mutation at position 243 were inserted into the expression vector $pSFFV_{neo}$ (provided by Dennis Loh, Washington University) (23). The cDNA was introduced into L6 cells on the third passage by using cationic liposomes (Lipofectin reagent, BRL). For selection, cells were cultured in medium containing 2 mg of G418 per ml (GIBCO). Colonies of resistant cells were isolated by using glass cloning cylinders (Bellco Glass). Stock cultures of the cell lines were maintained in medium containing 0.2 mg of G418 per ml. GLUT4 expression in the cell lines was found to be stable for at least 12 passages. Under appropriate conditions, cells from the lines used in this study fused to form myotubes. However, the results presented were obtained using myoblasts to avoid complications due to the endogenous GLUT4 gene, which is expressed at low levels in L6 myotubes.

Abbreviations: Ab, antibody; BSA, bovine serum albumin; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; IGF-1, insulin-like growth factor 1; Bt_2cAMP, $N^6,O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate.

GLUT1 and GLUT4 were transiently expressed in CHO cells by using a Sindbis virus expression system, which enables expression of large amounts of recombinant proteins in eukaryotic cells (24). The expression vector (KASind) was based on a full-length Sindbis virus cDNA clone (25) containing an additional subgenomic mRNA promoter (26) and an additional cloning site (27). Virus stocks were produced essentially as described (28). Briefly, capped genomic-length RNA transcripts were synthesized *in vitro* with SP6 RNA polymerase using the KASind plasmids linearized with *Mlu* I. For transfection, 10 μg of transcript RNA was mixed with 50 μg of Lipofectin and added to baby hamster kidney cells (10^8 cells per 15-cm plate). After 15 min at 37°C, the cultures were rinsed in solution A and then cultured in DMEM containing 7% fetal calf serum for 32 hr. The medium was then collected as viral stock and stored at −80°C. Thawed aliquots had titers of 10^8–10^9 plaque-forming units/ml.

Confluent CHO cells were passaged and seeded at one-third density in 24-well dishes (Falcon). After 15 hr of culture, the cells were rinsed with solution A containing 0.2% fetal calf serum and incubated for 25 min at 37°C with diluted viral stock (150 μl per well) at a multiplicity of infection of ≈50. Growth medium was then added and cells were incubated at 37°C. Depending on the virus used, GLUT1 or GLUT4 expression was markedly increased after 6 hr of infection, and approximately 50% and 10%, respectively, of the transporters were determined to be at the cell surface (27).

Detection of Glucose Transporters by Immunoblotting. Cells were rinsed twice with solution A, scraped from the dish, and homogenized in solution B (0.25 M sucrose/1 mM EDTA/10 mM NaHepes, pH 7.4; 1 ml per 10-cm dish). Samples of the homogenates were subjected to SDS/PAGE by the method of Laemmli (29). Proteins were electrophoretically transferred from the 10% acrylamide gels to nitrocellulose sheets. To identify GLUT4 and GLUT1, the sheets were incubated with polyclonal antibodies (Abs) against synthetic peptides having the sequence of the last 12 amino acids in the COOH termini of the respective transporters. Transporters were detected by using ^{125}I-labeled protein A and autoradiography (9).

Estimation of Glucose Transport Activity. Myoblasts were seeded into 24-well culture dishes (Falcon). After 4–6 days of culture, the cells were incubated for 5 hr at 37°C in DMEM without serum (18). Cells were then incubated with insulin (Lilly), insulin-like growth factor 1 (IGF-1; Kabi Peptide Hormones) or other additions for 20 min in medium composed of Krebs/Ringer phosphate buffer (135 mM NaCl/5.4 mM KCl/1.4 mM $CaCl_2$/1.4 mM $MgSO_4$/10 mM sodium phosphate, pH 7.4) containing 1% bovine serum albumin (BSA, crystalline; Sigma). To initiate uptake, the medium was replaced with medium (37°C) containing the same additions plus 50 μM 2-deoxy[^{3}H]glucose. Uptake was terminated by washing the cells three times with Krebs/Ringer phosphate buffer (2 ml of 0°C buffer per wash). The total time required for the wash was 5 s. NaOH (0.5 ml of 0.4 M) was added to each well to dissolve the cells. After neutralization

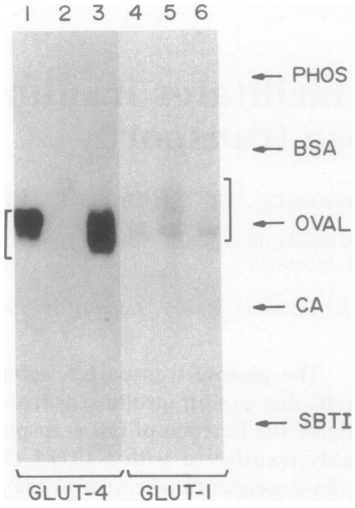

FIG. 1. Relative levels of mutant GLUT4 species in L6 cell lines. Samples (25 μg) of rat heart low-density microsomes (lanes 1 and 4) and homogenate samples (75 μg of protein) of L6 myoblasts (lanes 2 and 5) and 243-6 myoblasts (lanes 3 and 6) were subjected to SDS/PAGE. The proteins were then transferred to nitrocellulose. GLUT1 (lanes 4–6) and GLUT4 (lanes 1–3) were detected by using Ab against the COOH-terminal regions of the respective proteins and ^{125}I-labeled protein A. Photographs of autoradiograms are presented. Locations of the following protein standards are indicated: phosphorylase b (PHOS, M_r = 97,500), BSA (M_r = 68,000), ovalbumin (OVAL, M_r = 45,000), carbonic anhydrase (CA, M_r = 29,000), and soybean trypsin inhibitor (SBTI, M_r = 21,000). Note that because of the different reactivities of the two antisera, it is not appropriate to use the band intensities to compare the amount of GLUT4 relative to the amount of GLUT1.

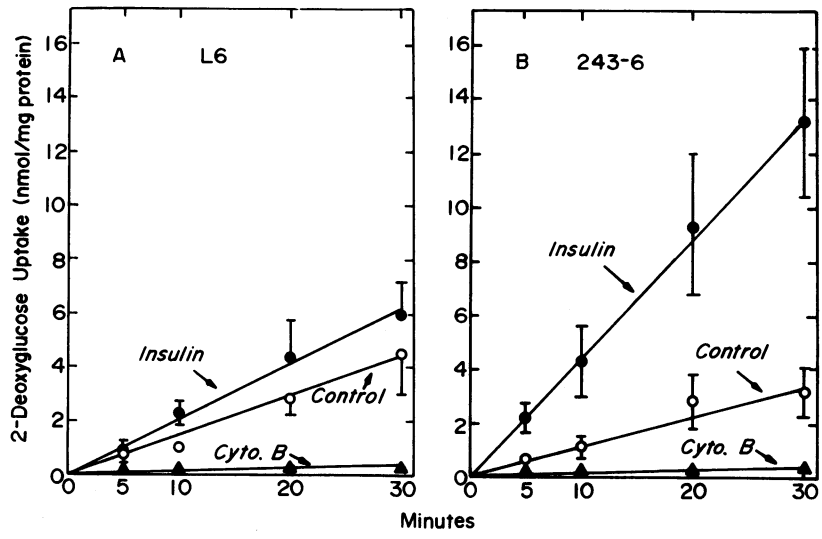

FIG. 2. Stimulation of 2-deoxy[^{3}H]glucose uptake by insulin in control L6 cells and in L6 cells overexpressing GLUT4. Control L6 cells and 243-6 cells were cultured for 4 days. The cells were incubated at 37°C for 20 min in Krebs/Ringer phosphate buffer containing 2% BSA and no other addition or either insulin (2.5 milliunits/ml) or cytochalasin B (Cyto. B, 10 μM). 2-Deoxy[^{3}H]glucose uptake was measured after increasing times of incubation. The results represent mean values ± SEM of three experiments.

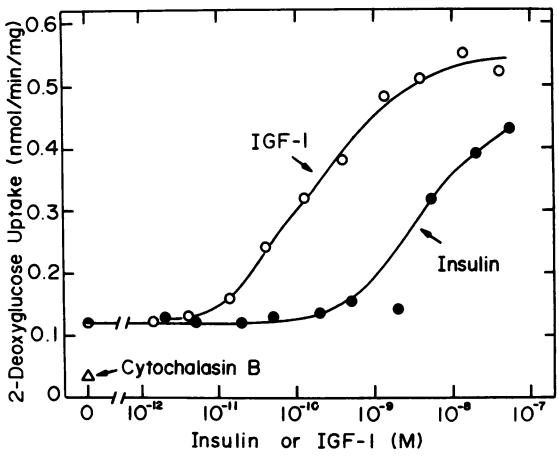

FIG. 3. Effect of increasing concentrations of insulin and IGF-1 on 2-deoxy[^{3}H]glucose uptake in cells overexpressing GLUT4. The 243-6 myoblasts were incubated for 20 min without additions, with 10 μM cytochalasin B, or with increasing concentrations of insulin or IGF-1 before uptake was measured after a 5-min incubation with 2-deoxy[^{3}H]glucose.

radioactivity was measured by scintillation spectroscopy. Values for uptake are expressed in terms of protein (30).

CHO cells were incubated with virus for 6 hr, rinsed with solution A, and incubated in DMEM containing 1% BSA and 20 μg of cycloheximide per ml for 40 min. The medium was then replaced with Krebs/Ringer phosphate [with or without 5 mM N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP)]. After 15 min 10 μM 2-deoxy[^{3}H]glucose was added and uptake was measured after 3 min as described above.

Preparation of Plasma Membrane Fragments (Lawns) and Detection of GLUT4 by Immunofluorescence Microscopy. Plasma membrane lawns were isolated using an adaptation of the method described by Moore *et al.* (31). Myoblasts were seeded on glass coverslips but otherwise cultured and treated with insulin and IGF-1 as described above. The cells were then rinsed with solution A and incubated for 30 s in solution A containing 0.5 mg of poly(L-lysine) per ml followed by three 5-s incubations in hypotonic buffer (23 mM KCl/1.7 mM $MgCl_2$/1 mM EGTA/and 10 mM Hepes, pH 7.5). The coverslips were submerged in solution C (70 mM KCl/5 mM $MgCl_2$/3 mM EGTA/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/30 mM Hepes, pH 7.5) and sonicated for 2 s at a power setting of 15 (Kontes 115-V disrupter) by using a 3.2 × 48 mm tapered probe centered 0.3 cm above the coverslip. Coverslips were then immediately immersed in solution C containing 2% paraformaldehyde. The lawns, which appeared by phase microscopy as small fragments attached to the coverslip, were washed to remove excess fixative. Coverslips were then incubated at 4°C for ≈18 hr with the GLUT4 Ab (50 μg/ml), washed, and incubated for 50 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ab (Cappel Laboratories). Next the specimens were washed and again fixed using paraformaldehyde before being incubated with an antiserum (1:100 dilution) to rat Na^+,K^+-ATPase (supplied by Robert Mercer, Washington University). After incubation with rhodamine-labeled goat anti-rabbit IgG (Cappel), the coverslips were washed and mounted in solution A supplemented with 10% glycerol and 1% *n*-propyl gallate (Sigma). Lawns were examined with a Zeiss Axioplan microscope equipped with a Bio-Rad MRC-500 laser confocal imaging system. Images of intact cells were obtained as described (16).

RESULTS

Overexpression of GLUT4 Potentiates Insulin/IGF-1 Stimulation of Glucose Transport. More than 50 cell lines were isolated after transfecting L6 myoblasts with GLUT4 cDNA. Although several of these expressed markedly increased levels of GLUT4 relative to normal L6 cells, none expressed

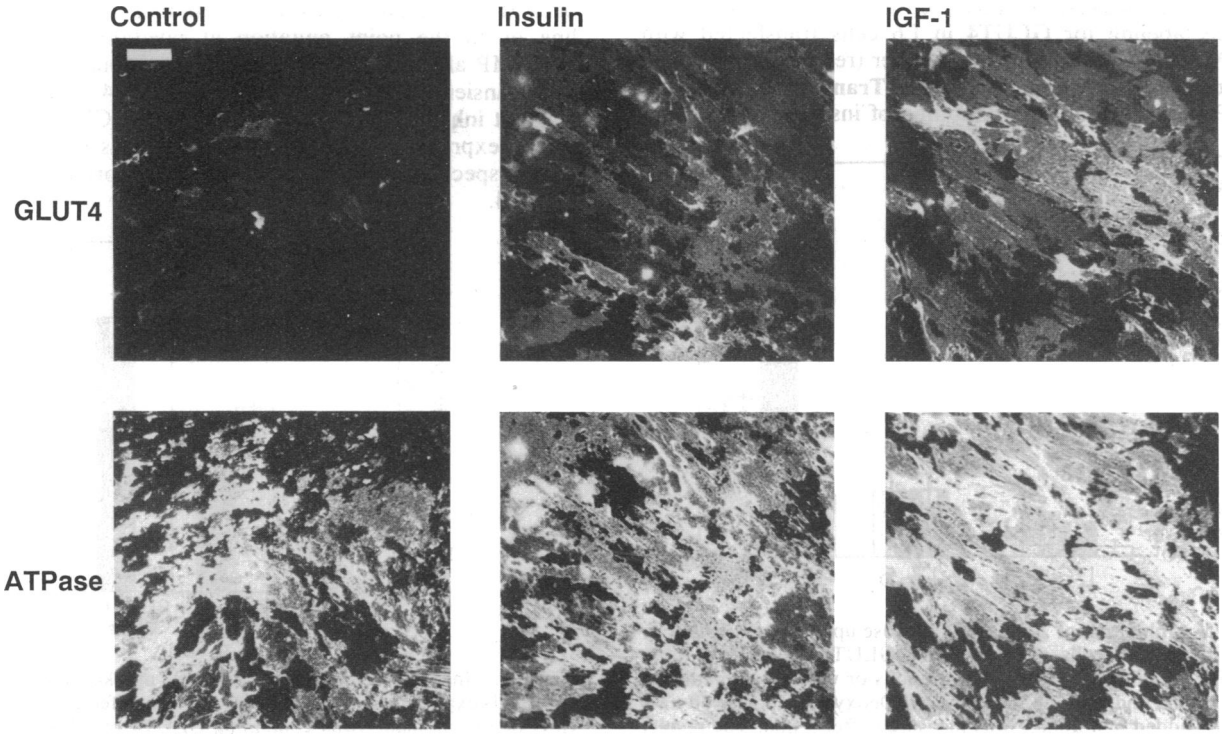

FIG. 4. GLUT4 labeling of plasma membrane lawns isolated from control cells and cells treated with insulin or IGF-1. The 243-6 cells were cultured on glass coverslips and incubated with either no additions (control), 1 μM insulin, or 100 nM IGF-1 for 30 min before plasma membrane lawns were prepared. Epifluorescent images were photographed using identical exposures for each fluorochrome. (Bar = 15 μm.)

the transporter at a level approaching that in muscle or fat cells. However, one clone (designated 243-6) having a point mutation at position 243 expressed transporter protein at a level comparable to that in rat heart (Fig. 1), which has the highest levels of GLUT4 of any tissue that has been examined (13). Levels of GLUT1 were comparable in normal L6 cells and 243-6 cells. Because of the extremely high level of GLUT4 expression, 243-6 cells were investigated further.

Glucose transport was assessed in normal L6 and 243-6 cells by measuring the rate of uptake of 2-deoxy[^{3}H]glucose. Uptake was linear for 30 min in cells from both lines (Fig. 2) and was almost completely inhibited by cytochalasin B, an inhibitor of glucose transport. The rate of uptake in the absence of insulin was not significantly different in L6 cells and 243-6 cells. However, the increment in 2-deoxy[^{3}H]-glucose uptake due to insulin was five times higher in the cells overexpressing GLUT4 than in the normal L6 cells.

IGF-1 also increased 2-deoxy[^{3}H]glucose uptake severalfold in 243-6 cells (Fig. 3), with the maximum effect representing an increase of ≈6-fold over basal. Insulin receptors have higher affinity for insulin than for IGF-1, and IGF-1 receptors bind IGF-1 with an affinity ≈50 times higher than that for insulin (32, 33). The half-maximal effect of IGF-1 was observed at a concentration of ≈0.15 nM. IGF-1 was ≈50 times more potent than insulin in increasing 2-deoxy[^{3}H]glucose uptake, suggesting that the stimulation of glucose transport was mediated by IGF-1 receptors.

Insulin and IGF-1 Stimulate Translocation of GLUT4 in 243-6 Cells. Immunofluorescence microscopy of GLUT4 labeling indicated a predominance of intracellular staining (results not shown) that was indistinguishable from that observed in other cells (23, 27, 35). Upon treatment of cells with insulin or IGF-1, we observed an increase in plasma membrane labeling for GLUT4 but no change in labeling of the Na^+,K^+-ATPase (Fig. 4). Quantitation of fluorescence from digitized images showed a 3-fold increase in GLUT4 at the cell surface. This insulin- or IGF-1-dependent movement of GLUT4 to the cell surface was not due to the mutation at position 243 because we observed a 3-fold increase in cell surface labeling for GLUT4 in L6 cells transfected with cDNA encoding wild-type transporter (results not shown).

Bt_2cAMP Inhibition of Glucose Transport. Increasing cAMP is associated with inhibition of insulin-sensitive glucose transport in cells that express GLUT4 *in vivo*. To investigate the possibility that the inhibitory effect was mediated specifically by the GLUT4 isoform, the effects of Bt_2cAMP on 2-deoxy[^{3}H]glucose uptake were assessed in L6 cells and 243-6 cells (Fig. 5). Bt_2cAMP had little if any effect on 2-deoxy[^{3}H]glucose uptake in L6 cells or in cells from line 243-5 (Fig. 5), a cell line that expresses low levels of GLUT4. In 243-6 cells incubated without insulin, Bt_2cAMP produced a small increase in 2-deoxy[^{3}H]glucose uptake; however, Bt_2cAMP decreased insulin-stimulated 2-deoxy[^{3}H]glucose uptake by ≈60% (Fig. 5). Similar effects of insulin and Bt_2cAMP were observed in an experiment in which 3-*O*-[^{3}H]methylglucose uptake was measured, and Bt_2cAMP was also found to inhibit IGF-1-stimulated transport in 243-6 cells by ≈60% (results not shown). Under the conditions of these experiments, sodium butyrate (2 mM) was without effect on 2-deoxy[^{3}H]glucose uptake (results not shown), consistent with the interpretation that Bt_2cAMP acted by mimicking an increase in intracellular cAMP.

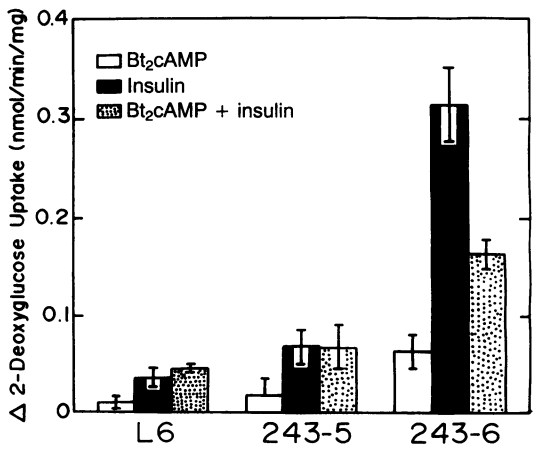

FIG. 5. Inhibition of 2-deoxy[^{3}H]glucose uptake by Bt_2cAMP in insulin-stimulated cells overexpressing GLUT4. Cells were incubated for 20 min at 37°C without additions or with 2.5 milliunits of insulin per ml and/or 2 mM Bt_2cAMP. 2-Deoxy[^{3}H]glucose (50 μM) was then added and uptake was measured after 5 min. The results represent the increments over uptakes observed in the absence of insulin and Bt_2cAMP and are mean values ± SEM from five experiments. Cell line 243-5 was generated at the same time as 243-6 but expresses approximately one-tenth the level of GLUT4.

GLUT4 and GLUT1 can be overexpressed in CHO cells by using the Sindbis RNA viral expression system (27). Uptake of 2-deoxy[^{3}H]glucose was increased by 2-fold and 3-fold in CHO cells overexpressing GLUT4 and GLUT1, respectively (Fig. 6). These increases in uptake were due to expression of the transporters as opposed to viral infection *per se* because there was no significant increase in uptake in CHO cells infected with control virus. Bt_2cAMP markedly inhibited 2-deoxy[^{3}H]glucose uptake in cells expressing GLUT4 but was without effect in mock-infected cells or in cells overexpressing GLUT1.

DISCUSSION

Overexpression of GLUT4 in L6 cells resulted in two major functional changes in glucose transport. IGF-1/insulin-stimulated glucose transport was increased severalfold (Figs. 2, 3, and 5), and stimulated transport became sensitive to inhibition by cAMP derivatives (Fig. 5). The inhibitory effect of cAMP was not due to clonal selection of an atypical L6 cell line or to the point mutation at position 243, because Bt_2cAMP also inhibited transport in a population of CHO cells transiently expressing wild-type GLUT4. That transport was not inhibited in normal L6 cells or in CHO cells transiently expressing GLUT1 strongly suggests that increased cAMP specifically inhibits glucose transport mediated by GLUT4.

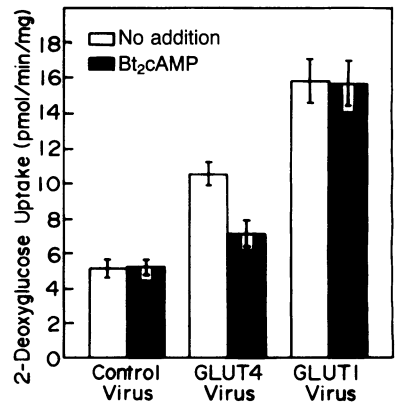

FIG. 6. Inhibition of 2-deoxy[^{3}H]glucose uptake by Bt_2cAMP in CHO cells expressing GLUT4. CHO cells were infected with control virus or recombinant virus containing GLUT4 or GLUT1 cDNA. After 6 hr the cells were incubated with cycloheximide for 45 min. Cells were then incubated (± 5 mM Bt_2cAMP) for 15 min before 2-deoxy[^{3}H]glucose uptake was measured. The values presented are mean values ± SD from four cultures.

Most, if not all, of the effects of cAMP in cells are thought to involve activation of cAMP-dependent protein kinase. It is interesting to note that GLUT4 can be phosphorylated *in vitro* by this kinase (9). Furthermore, although GLUT1 (8, 34) and GLUT4 (9) are phosphorylated in fat cells, only GLUT4 phosphorylation is increased in response to agents that increase intracellular cAMP. The present findings are consistent with the hypothesis that phosphorylation of GLUT4 accounts for the inhibition of insulin-stimulated glucose transport by cAMP.

Expression of GLUT4 in 243-6 cells led to a marked increase in IGF-1/insulin-stimulated glucose transport, consistent with the key role of GLUT4 in mediating insulin-stimulated transport *in vivo*. The exclusion of GLUT4 from the plasma membrane in basal cells is one factor that contributes to the striking increase in this transporter at the cell surface following treatment with insulin (35, 36). A second factor is the ability of GLUT4 to move to the plasma membrane in response to insulin. Previous investigations of GLUT4 translocation have relied on differential centrifugation to isolate plasma membranes and intracellular membranes. However, adequate separation of these membranes for studying GLUT4 translocation in myoblasts and fibroblasts has been difficult. Therefore, we used a different method involving sonication of cells to generate plasma membrane fragments. By deep-etch freeze-fracture electron microscopy, these fragments, which remain attached to the substratum, appear to contain only elements that are characteristic of the plasma membrane, such as clathrin-coated pits, caveoli, and cytoskeletal attachments (31). The levels of GLUT4 in these fragments were increased severalfold after IGF-1/insulin treatment (Fig. 4), suggesting that translocation of GLUT4 to the plasma membrane accounts for the increase in the IGF-1/insulin-stimulated component of 2-deoxy[^{3}H]glucose uptake observed in the transfected cells.

The magnitude of the hormone-stimulated increase in cells expressing only GLUT1 is limited by the high levels of this transporter that are present in the plasma membrane even in the absence of insulin. This limitation probably explains why insulin/IGF-1 elicited a relatively small increase in glucose transport in the normal L6 myoblasts, which express GLUT1 (20, 21). However, expression of GLUT4 *per se* is not sufficient to produce insulin-stimulated transport in all cells. Insulin was unable to stimulate transport in 3T3 L1 fibroblasts overexpressing GLUT4 (23). Untransfected 3T3 L1 fibroblasts and L6 myoblasts are similar in that both express GLUT1. A difference between these cell types is that insulin stimulates glucose transport by as much as 2-fold in L6 cells, presumably due to translocation of GLUT1 (20), whereas insulin does not stimulate transport or translocation of GLUT1 in 3T3 L1 fibroblasts (37). In L6 cells GLUT4 may utilize the same vesicular transport system that moves GLUT1 to the plasma membrane in response to insulin. Alternatively, there might be unknown features of the L6 cells that enable GLUT4 to induce components required for its translocation.

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