Insulin-sensitive Association of GLUT-4 with Endocytic Clathrin-coated Vesicles Revealed with the Use of Brefeldin A*

(Received for publication, April 29, 1993, and in revised form, November 18, 1993)

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The interaction of the adipocyte/skeletal muscle glucose transporter (GLUT-4) with clathrin lattices may be important in maintaining its intracellular distribution. To better understand the role of clathrin lattices in the sorting of GLUT-4, we have attempted to determine the cellular origin of clathrin-coated vesicles (CCVs) that contain this transporter. The fungal toxin brefeldin A (BFA) causes the selective disassembly of clathrin lattices at the trans-Golgi network (TGN), but not at the plasma membrane (PM), thus providing a way of estimating the proportion of GLUT-4 in PM- versus TGN-derived clathrin lattices. Exposure of 3T3-L1 adipocytes to BFA resulted in a rapid disassembly of clathrin lattices at the TGN, observed by optical sectioning microscopy, and to a pronounced decrease in the yield of CCVs purified from these cells. Thus, CCVs isolated from BFA-treated cells are likely to be derived from the PM. Immunoblotting experiments revealed the presence of GLUT-4 in such CCVs, suggesting that under basal conditions the transporter is continually retrieved from the PM through the CCV pathway. Exposure of both BFA-treated or non-treated cells to insulin resulted in a 4–6-fold increase in the concentration of GLUT-4 at the PM. In parallel, the concentration of GLUT-4 in PM-derived CCVs decreased by 60%. These results suggest (a) that the effect of insulin to increase the cell surface concentration of GLUT-4 is not inhibited by BFA, and (b) that a decreased association of GLUT-4 with endocytic CCVs may be important in facilitating its increased cell surface concentration in response to the hormone.

The effect of insulin to increase glucose uptake by adipose cells involves the redistribution of a specific glucose transporter isoform, GLUT-4, from a principally intracellular location to the plasma membrane (PM) (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). The cellular and molecular mechanisms involved in the cellular sorting of GLUT-4 are not completely understood. Electron microscopy analysis has shown that GLUT-4 associates with structures of the endocytic pathway, including clathrin-coated pits at the plasma membrane (Robinson et al., 1992). In addition, recent experiments from our laboratory indicate that the transporter is present in purified clathrin-coated vesicles derived from isolated rat adipose cells. These morphological and biochemical results suggest that the interaction of GLUT-4 with clathrin lattices may be part of the mechanism that maintains the intracellular distribution of transporters. Clathrin lattices may contribute to maintaining the intracellular localization of GLUT-4 in two ways. First, the transporter may interact with clathrin lattices formed at the trans-Golgi network (TGN), which may directly or through a recycling mechanism lead to intracellular retention of transporters. Second, the interaction with clathrin lattices at the plasma membrane may lead to a high rate of retrieval of GLUT-4 from the cell surface, resulting in a principally intracellular localization at steady state.

The fungal toxin brefeldin A causes the complete dispersion of Golgi-derived clathrin lattices but does not affect the assembly or function of PM-derived clathrin-coated vesicles (Robinson and Kreis, 1992; Wong and Brodsky, 1992). The selective elimination of Golgi-derived clathrin coats by BFA provides a way of estimating the proportion of GLUT-4 that resides in Golgi-derived versus PM-derived clathrin-coated vesicles purified from adipose cells, and the effects of insulin on the interaction of transporters with such structures. Analysis of the effects of BFA on cellular trafficking pathways has also revealed the existence of at least three endomembrane systems within the cell, consisting of an ER-Golgi system, a post-Golgi-TGN-endosomal system and a prelysosomal-lysosomal system (Lippincott-Schwartz et al., 1991). Forward transport out of these systems is inhibited by BFA, but traffic within the systems is unaffected (Klausner et al., 1992). Thus, the analysis of insulin action in BFA-treated cells can help to delineate whether the changes in GLUT-4 sorting which occur in response to this hormone involve one or more independent endomembrane systems. In this paper, the effects of BFA on the subcellular distribution of GLUT-4 on its association with purified clathrin-coated vesicles and on basal and insulin-stimulated glucose uptake are reported.

MATERIALS AND METHODS

Cells—3T3-L1 fibroblasts (American Type Culture Collection) were seeded and fed every 2 days in Dulbecco's modified Eagle's medium supplemented with 0.75 mg/ml glutamine, non-essential amino acids (Life Technologies, Inc.), 50 units/ml penicillin, 50 μg/ml streptomycin, and 1% fetal calf serum (UBI), and grown under 10% CO₂. At confluence, differentiation was started by exposure of cells to culture medium containing 0.25 μM dexamethasone (Sigma), 0.5 μM 2,4-dimethylmedium containing 0.25 μM dexamethasone (Sigma), 0.5 μM isobutylmethylxanthine (Sigma), and 1 μM insulin (porcine crystalline, Lilly). After 48 h, this was replaced with fresh medium containing 1 μM insulin, which was removed after 48 h. Lipid droplets were observed in 90–95% of the cells 4 days after initiating differentiation. After 10 days of differentiation, cells were treated with BFA (Sigma) or insulin for the indicated times and at the indicated concentrations.

Immunofluorescence—Cells were seeded and differentiated on glass coverslips. After treatment with BFA at 37 °C, monolayers were placed on ice, washed rapidly twice with cold phosphate-buffered saline, and fixed by immersion in ~20 °C methanol for 6 min. The methanol was
removed, and coverslips were briefly air dried before immersion in phosphate-buffered saline containing 1% fetal calf serum. After 30 min, coverslips were stained with anti-clathrin heavy chain monoclonal antibody Chc5.9 (ICN) at a final concentration of 10 μg/ml. Primary antibody was detected using a rhodamine-conjugated goat anti-mouse IgM (Tago). Samples were visualized on a Zeiss IM-35 microscope, using a Nikon Interphase lens for image deconvolution; 35 serial two-dimensional images were recorded at 0.2-μm intervals using a thermoelectrically cooled charge-coupled device camera (Photometrix Ltd., Tuscon, AZ). Each image was corrected for lamp intensity variations and photobleaching. Blurring of fluorescence from regions above and below the plane of focus was reversed using an iterative reconstruction algorithm based on the theory of ill-posed problems (Car Hag, 1990).

Subcellular Fractionation—Cells were seeded and differentiated on 150-mm dishes, and fractionation was done using previously characterized procedures (James et al., 1989). Briefly, after the indicated treatments, each dish was placed on ice and scraped into 25-ml ice-cold TES buffer (10 mM Tris, 1 mM EDTA, 250 mM sucrose, 1 mM 1,10-phenanthroline, 10 μM leupeptin, and 1 mM benzamidine), pH 7.4. Cells were homogenized with 15 strokes of a motor-driven Teflon glass homogenizer and centrifuged at 16,000 × g for 20 min in a Beckman JA-17 rotor. The supernatant was centrifuged at 200,000 × g for 60 min in a Beckman Ti-50.2 rotor to obtain a pellet (intracellular membranes). The pellet from the 16,000 × g spin was resuspended in 6 ml of TES buffer with 10 strokes of a glass Dounce homogenizer and was layered on the top of a 1.12 M sucrose cushion made in TES. After centrifugation at 100,000 × g for 1 h in a Beckman SW-28 rotor, the interphase was collected, diluted with 20 ml of TES, and centrifuged for 15 min at 200,000 × g in a Beckman Ti-50.2 rotor. The resulting pellet (plasma membranes) was resuspended in TES.

Clathrin-coated Vesicle Preparation—Coated vesicles were isolated by the differential centrifugation procedure described by Campbell et al. (1984). We adapted this method to obtain a more rapid preparation of coated vesicles from smaller amounts of tissue (Corvera et al., 1990). Two 150-mm plates of 3T3-L1 adipocytes were employed per condition. Cells were scraped and homogenized in 1 ml/plate of ice-cold buffer composed of 100 mM MES, 1 mM EDTA, 0.5 mM magnesium chloride, 0.02% sodium azide, 50 mM sodium fluoride, 100 μM sodium vanadate, 1 mM 1,10-phenanthroline, 10 μM leupeptin, and 1 mM benzamidine adjusted to pH 6.5, using three 30-s bursts of a Polytron tissue grinder set at 80% of maximal speed. The homogenates were made 0.05% with Triton X-100, vortexed, placed in Beckman microcentrifuge polyallomer tubes, and centrifuged for 12 min at 15,600 × g in a Beckman TLA 100.3 rotor. The supernatant, beneath the fat cake, was removed and centrifuged for 21 min at 36,350 × g. The resulting pellet was resuspended in 100 μl of buffer, and an equal volume of a solution composed of 12.5% (w/v) sucrose and 12.5% (w/v) Ficoll (Pharmacia LKB Biotechnology, Inc.) was added. After vortexing, the suspension was centrifuged for 2 min at 36,350 × g, and the supernatant was diluted with 5 volumes of ice-cold buffer and centrifuged for 21 min at 72,000 × g. The pellet from this centrifugation was 80% enriched in clathrin-coated vesicles.

Electrophoresis and Immunoblotting—Aliquots (50–100 μg of protein) of each fraction were electrophoresed on 10% polyacrylamide gels and electroelutiontransferred onto nitrocellulose paper. Blots were probed with anti-GLUT-4 carboxyl-terminal polyclonal antibody (East Acres Biologicals) or anti-clathrin heavy chain monoclonal antibody Chc5.9 (ICN). The primary antibodies were detected with polyclonal goat anti-rabbit immunoglobulins coupled to horseradish peroxidase (Amersham Corp.) and developed with enhanced chemiluminescence. The intensity of the bands was quantified using an LKB Ultroscan XL laser densitometer. Standard curves of intracellular microsomes were run periodically to ensure that signals obtained from each fraction were within a linear range.

RESULTS

It has been shown recently that BFA causes the disassembly of Y-adaptin and of TGN-derived clathrin lattices (Robinson and Kreis, 1992; Wong and Brodsky, 1992). To effect this, BFA was preincubated with adipocytes and cell-specific. We investigated whether 3T3-L1 adipocytes would also respond to BFA with the disassembly of TGN-derived clathrin lattices. Immunofluorescence analysis of 3T3-L1 cells was hampered by the general morphology of adipocyte cells, which form tightly packed monolayers, and are rounder and thicker than other cells due to the accumulation of large lipid droplets. To obtain images that would more accurately reflect the distribution of clathrin in different regions of these cells, we employed optical sectioning microscopy and image reconstruction.

Immunofluorescence microscopy of cells stained with anti-clathrin heavy chain monoclonal antibody Chc5.9 reveals a characteristic pattern of both peripheral and juxtanuclear punctate staining (Fig. 1, left column, top panel). The juxtanuclear staining is quite pronounced, suggesting that TGN-derived clathrin-coated vesicles are abundant in these cells. Within 10 min of treatment of the cells with 10 μg/ml BFA, the staining in the juxtanuclear region decreased dramatically, and changed to a diffuse cytoplasmic pattern (Fig. 1, left column, center panel). However, optical sections through the middle of the cell revealed some clathrin-coated vesicles remaining in the perinuclear region (Fig. 1, center column, center panel). Treatment with 100 μg/ml BFA for 10 min resulted in a complete disappearance of the perinuclear staining (Fig. 1, left and center columns, bottom panels). Under these conditions, the toxin had no effect on the density or appearance of peripheral clathrin-coated structures (Fig. 1, right column), and endocytosis was unimpaired as assessed by the ability of the cells to internalize transferrin receptors (not shown). The concentration of the toxin required to produce the observed effects in 3T3-L1 adipocytes within 10 min is relatively high compared with the concentrations required in other cells. This may relate to the high lipid content of adipocytes and the lipophilic nature of the toxin; partitioning of BFA into the lipid vacuoles may reduce its effective concentration in the cytosol. Consistent with this possibility is the observation that a complete dispersion of TGN clathrin lattices could be observed with low doses of BFA when incubations were prolonged to 30–40 min (not illustrated).

Having established that 3T3-L1 adipocytes respond to BFA with a disruption of TGN-derived clathrin lattices, we then investigated the actions of this toxin on the subcellular distribution of GLUT-4, and on the effects of insulin on this distribution. Adipocytes were treated with or without 100 μg/ml BFA for 10 min and then with or without insulin for a further 10 min. PMs and intracellular microsomal membranes (IMs) were then isolated and analyzed by polyacrylamide gel electrophoresis and immunoblotting with anti-GLUT-4 polyclonal anti-
Fig. 1. Effect of BFA on the distribution of clathrin heavy chain in 3T3-L1 adipocytes. Cells were grown to confluence and differentiated for 10 days on glass coverslips. BFA was added at the indicated concentration for 10 min, and cells were then fixed and stained with anti-clathrin heavy chain monoclonal antibody Chc5.9, followed by a rhodamine-conjugated anti-IgM-specific second antibody. Nonrestored two-dimensional images are shown on the left. After restoration single optical planes from the middle of the cell (Perinuclear) or from the cell surface (Surface) were photographed. Arrows indicate the presence of punctate structures in the perinuclear or cell surface regions. Bar, 5 μm.

serum. Because BFA has profound effects on the functional properties of endomembrane systems, we first examined its effects on the yield and purity of isolated PMs and IMs. We could not detect a significant effect of BFA on the yield of PMs or IMs (not shown), nor on the gross polypeptide profile (Fig. 2) nor on the enrichment of the Na+/K+ ATPase in isolated PMs (not shown). Incubation of cells with 10 μM insulin caused a 4–5-fold increase in the concentration of GLUT-4 in isolated PMs, and a concomitant decrease in the concentration of transporter in IMs (Fig. 3, compare C and I). Treatment with BFA alone caused a smaller 1.5–2-fold increase in the concentration of GLUT-4 in the PM fraction, and a decrease in its concentration in IMs (Fig. 3, compare C and BFA). Interestingly, subsequent treatment of BFA-treated cells with insulin resulted in a further increase in the cell surface concentration of GLUT-4, to the level elicited by insulin in the absence of prior BFA treatment (Fig. 3, compare I and BFA+I).

The specificity of the effects of BFA to increase the cell surface concentration of GLUT-4 were assessed by examining the effects of the toxin on GLUT-1. This transporter has been estimated to be approximately three times more abundant than GLUT-4 in fully differentiated cells and is partitioned between plasma membranes and low density intracellular membranes (Piper et al., 1991). Stimulation of cells with insulin resulted in a 1.5–2-fold increase in the amount of GLUT-1 in the PM (Fig. 4, compare C and I). We did not detect a significant increase in the plasma membrane concentration of GLUT-1 in response to BFA (Fig. 4, compare C and BFA), but insulin was still able to increase the cell surface concentration of transporter after treatment with the toxin (Fig. 4, compare I and BFA+I). These

Fig. 2. Effects of BFA and insulin on the polypeptide composition of plasma membranes and intracellular membranes. 3T3-L1 cells were grown and differentiated for 10 days and treated without (−) or with (+) 100 μg/ml BFA (BFA) for 10 min and then without (−) or with (+) 10 μM insulin (I) for another 10 min. Plasma membranes and intracellular microsomal membranes were then prepared, and 50 μg of protein from each fraction analyzed by electrophoresis and Coomassie Blue staining. The position of molecular weight markers is indicated.
The GLUT-4 signal was detected as a relatively broad band of protein, respectively. Shown are immunoblots and densitometric scans from a representative experiment, which was repeated three times with similar results. The apparent decrease in the concentration of GLUT-1 in the intracellular membranes in response to BFA was not reproducible from experiment to experiment. Results confirm that the ability of insulin to modulate the distribution of glucose transporters is unaffected by BFA. Whereas it is possible that a small effect of BFA on GLUT-1 would be undetectable in our assay, it is also possible that BFA may impact selectively on mechanisms that specifically operate on GLUT-4 and not GLUT-1.

We performed experiments to determine whether GLUT-4 would be present in clathrin-coated vesicles purified from 3T3-L1 adipocytes. Coated vesicles obtained by differential centrifugation as described under "Materials and Methods" contain 80-100-nm spheroid structures displaying the pentagonal and hexagonal network characteristic of clathrin-coated vesicles, smooth vesicles and filaments, and a small amount of amorphous material. To separate clathrin-coated structures from the smooth vesicle contaminants, we further purified the preparation by electrophoresis in non-sieving agarose gels. Clathrin-coated vesicles were isolated and electrophoresed on two lanes of a 0.15% agarose gel. One lane was directly transferred onto nitrocellulose, and the other sliced as indicated by the numbers in Fig. 5A. The nitrocellulose blot was then probed with anti-GLUT-4 rabbit antiserum, stripped, and reprobed with a monoclonal antibody against the clathrin heavy chain. The GLUT-4 signal was detected as a relatively broad band which migrated toward the anode. The clathrin signal was detected in a narrow, slower migrating band and predominately in a relatively broad, faster migrating band. The nature of the slow migrating component is not known, but may consist of empty clathrin cages which are frequently observed by negative staining. A complete co-migration of GLUT-4 with the major clathrin band was observed (Fig. 5A).

The polypeptide composition of the structures separated on the agarose gel was analyzed by polyacrylamide gel electrophoresis and silver staining of the gel slices (Fig. 5B). The only prominent polypeptides found in the fractions containing GLUT-4 were the 180-kDa heavy chain of clathrin (Fig. 5B, Chc), the 100–115-kDa assembly polypeptide complexes (AP), and the 32–35-kDa clathrin light chains (Clt). Negative staining of this fraction revealed a marked predominance of coated vesicles (Fig. 5C, right panel), with the vast majority of the smooth vesicle contaminants and aggregates remaining in fractions closer to the origin of the agarose gel (Fig. 5C, left panel). Thus, clathrin-coated vesicles separated well from contaminants in the preparation. The complete co-migration of GLUT-4 with clathrin (Fig. 5A), indicates that in this preparation, GLUT-4 is contained in clathrin-coated vesicles and not in smooth vesicle contaminants.
Fig. 5. Co-migration of GLUT-4 with clathrin-coated vesicles on non-sieving agarose gels. A, clathrin-coated vesicles were isolated, and approximately 50 μg of protein was separated by electrophoresis on a 0.15% agarose gel. After 24 h, the gel was directly transferred to nitrocellulose paper and probed with anti-GLUT-4 antiserum (anti-GLUT-4). The blot was then stripped and reprobed with a monoclonal antibody against the clathrin heavy chain (anti-CLATHRIN). The origin of the gel is indicated by the arrow. Migration was toward the anode. B, clathrin-coated vesicles were isolated, and approximately 150 μg of protein was separated by electrophoresis on a 0.15% agarose gel. After 24 h, the gel was sliced into five fractions as indicated by the numbers in A. The slices were lyophilized and analyzed by polyacrylamide gel electrophoresis and silver-staining. The expected position of the clathrin heavy chain (Chc), the 100–120-kDa assembly polypeptides (AP), and the 33–35-kDa clathrin light chains (Clc) are indicated. The asterisk represents a band which contaminated all the lanes which probably correspond to BSA. The positions of standard molecular weight markers on the gel are indicated. C, agarose gel slices corresponding to slice 1 and 5 in A were homogenized by aspiration through a 22-gauge needle, and aliquots were placed on Formvar/carbon-coated copper grids. Samples were negatively stained with 1% uranyl acetate and observed on a Jeol 100-S electron microscope. We repeatedly observed a layer of amorphous material in the background of the grid, which appears to correspond to components of the agarose released by the homogenization procedure. However, both non-coated and clathrin-coated vesicles could be clearly distinguished over this background.
clathrin-coated vesicles were isolated as described under “Selective elimination by BFA of TGN-derived clathrin lattices.” An equal amount of coated vesicle protein (50 μg) obtained from non-treated or BFA-treated cells was separated on a 0.15% agarose gel and analyzed by immunoblotting with anti-clathrin antibody. Thus, the decrease of coated vesicles from BFA-treated cells is likely to occur as a consequence of the disruption of TGN-derived lattices. An equal amount of coated vesicle protein (50 μg) obtained from non-treated or BFA-treated cells was separated on a 0.15% agarose gel and analyzed by immunoblotting with anti-GLUT-4 antibody (Fig. 6). GLUT-4 was readily detected in clathrin-coated vesicles obtained both from control and BFA-treated cells, indicating that the transporter is present in PM-derived vesicles. The concentration of GLUT-4 was slightly lower in coated vesicles isolated from BFA-treated cells, suggesting that the concentration of transporter is higher in TGN-derived vesicles than in PM-derived clathrin lattices. The presence of GLUT-4 in clathrin-coated vesicles derived from the plasma membrane suggests that internalization of GLUT-4 occurs continually, in the absence of insulin stimulation. This finding is consistent with previous observations of GLUT-4 in clathrin lattices at the plasma membrane (Robinson et al., 1999).

Quantitative analysis of the yield of clathrin-coated vesicles from BFA-treated cells suggests that approximately 50% of the total cellular GLUT-4 can be found in endocytic clathrin-coated vesicles at steady state. This is a significant amount, given the rapid transit time of proteins through the endocytic pathway and the short half-life of clathrin-coated vesicles.

We examined the effects of insulin on the association of GLUT-4 with clathrin lattices. The coated vesicle pellet obtained from insulin and/or BFA-treated cells was resuspended in 100 μl of buffer and analyzed by polyacrylamide gel electrophoresis and immunoblotting. Treatment with insulin for 10 min caused a 40% decrease in the concentration of GLUT-4 in clathrin-coated vesicles (Fig. 7, compare lanes 3 and 4). The insulin-induced decrease in GLUT-4 in this preparation is due to an actual decrease in the concentration of GLUT-4 within these structures because the yield of clathrin-coated vesicles was unchanged by insulin treatment (results not shown). We used BFA to determine whether the decrease in transporter concentration was occurring in endocytic or TGN-derived clathrin lattices. Exposure of cells to BFA alone resulted in a decrease of 50–60% in the amount of GLUT-4 in isolated clathrin-coated vesicles, measured by densitometric analysis of the bands from four independent experiments (Fig. 7, compare lanes 1 and 3). This effect can be explained by the 50% decrease in the yield of clathrin-coated vesicles that occurs in response to BFA and by the slightly lower concentration of GLUT-4 in the remaining vesicles (Fig. 6). These results suggest that under control conditions, 40–50% of clathrin-coated vesicle-associated GLUT-4 resides in the vesicle population derived from the plasma membrane.

Exposure of BFA-treated cells to insulin resulted in a pronounced 60–70% decrease in the amount of GLUT-4 in isolated clathrin-coated vesicles (Fig. 7, compare lanes 1 and 2). The comparison of the insulin-induced decrease in the concentration of GLUT-4 in clathrin-coated vesicles obtained before or after BFA treatment (40 vs. 60–70% decrease) together with the information on the relative yield of clathrin-coated vesicles (50% decrease in response to BFA) and of the concentration of GLUT-4 in these vesicles (10–20% lower in vesicles derived from BFA-treated cells), are consistent with a model in which insulin causes a 60% decrease in the concentration of transporter in PM-derived clathrin-coated vesicles, and a 30% decrease in its concentration in TGN-derived structures.

We analyzed whether the effects of BFA and insulin on GLUT-4 subcellular distribution were reflected by parallel effects on glucose transport activity. For these experiments, 3T3-L1 cells were treated without or with BFA for 10 min, and then with or without insulin for another 10 min. The uptake of 2-deoxyglucose was then assessed. Interestingly, BFA alone significantly inhibited basal glucose uptake to 40% of the value observed in control cells, with a half-maximal effect being observed at 10 μg/ml (Fig. 8, left panel). Under these conditions, the amount of cell surface GLUT-4 is increased by approximately 2-fold (Fig. 2). Thus, BFA appears to inhibit up to 80% of the basal activity of cell surface transporters. Insulin caused an approximately 8–9-fold increase in glucose transport activity in both BFA-treated and non-treated cells (Fig. 8, compare left and right panels). These results confirm that despite its...
BFA to increase the cell surface concentration of GLUT-4 and by the ability of insulin to further increase the cell surface concentration of transporter by 4–6-fold in BFA-treated cells (Fig. 3). Clearly, a significant amount of insulin-sensitive GLUT-4 must reside in endomembrane systems that are not functionally affected by the toxin, such as the endosomal-recycling pathway (Lippincott-Schwartz et al., 1991; Klausner et al., 1992). Such systems may be composed of endocytic clathrin lattices, small smooth vesicles, tubulovesicular bodies, and elements of the TGN which contain GLUT-4 (Slot et al., 1991a, 1991b).

The rapid effect of insulin to increase the plasma membrane concentration of GLUT-4 must result from rapid changes in the kinetics of GLUT-4 trafficking within the endosomal-recycling system. Our results show that insulin causes a >60% decrease in the concentration of GLUT-4 in clathrin-coated vesicles derived from the plasma membrane. Because we do not know what the rate-limiting steps are in the process of transporter internalization and recycling, it is not possible to determine what rate constants are altered by insulin to result in this effect. However, several hypotheses can be suggested. For example, insulin might increase the rate of uncoating of coated vesicles enriched in GLUT-4 and decrease the time between internalization of the transporter and its recycling to the plasma membrane. Alternatively, insulin might decrease the rate of sequestration of GLUT-4 into coated pits at the plasma membrane, leading to a decrease in the time between plasma membrane insertion of transporter and its reinternalization. Interestingly, recent kinetic experiments by Jhun et al. (1992) indicate that insulin causes a net decrease in the rate of endocytosis of GLUT-4. In addition, Yang and Holman (1993) and Czech and Buxton (1993) failed to observe an increase in the rate of GLUT-4 internalization in response to insulin. A proportional, large increase in the rate of GLUT-4 internalization might be expected to accompany the 15–20-fold increase in transporter concentration at the plasma membrane caused by the hormone, if the internalization pathway for GLUT-4 is not saturated. Taken together these results are consistent with the possibility that the decrease in the concentration of GLUT-4 in coated vesicles derived from the plasma membrane may reflect a decrease in the rate of sequestration of GLUT-4 into the endocytic pathway.

It is interesting that BFA inhibits glucose transport activity by 40–80%. BFA decreased transport activity in insulin-stimulated cells, which is mainly due to GLUT-4, and also inhibited basal glucose transport activity in 3T3-L1 fibroblasts, which is due to GLUT-1 (data not shown). Thus, the inhibitory effect of BFA is not isoform-specific. The half-maximal concentration of BFA required to inhibit glucose transport was higher than that required to disperse coat proteins, and thus may be the result from the interaction of the toxin with other targets. It is possible that BFA may directly interact with the transporter molecule to inhibit its intrinsic catalytic activity. In this regard it is interesting that both BFA and cytochalasin B are large carbon ring lactones containing hydroxyl groups. The latter is a potent and specific inhibitor of α-glucose transport.

Acknowledgments—The use of instrumentation of the Biomedical Imaging Group of the University of Massachusetts Medical School and the advice of Fred Fay and Doug Bowman for the morphological studies are gratefully acknowledged.

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