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IDENTIFICATION OF THE D-GLUCOSE-INHIBITABLE CYTOCHALASIN B BINDING SITE AS THE GLUCOSE TRANSPORTER IN RAT DIAPHRAGM PLASMA AND MICROSOMAL MEMBRANES

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[^{3}H]Cytochalasin B binding and its competitive inhibition by D-glucose have been used to identify the glucose transporter in plasma and microsomal membranes prepared from intact rat diaphragm. Scatchard plot analysis of [^{3}H]cytochalasin B binding yields a binding site with a dissociation constant of roughly 110 nM. Since the inhibition constant of cytochalasin B for D-glucose uptake by diaphragm plasma membranes is similar to this value, this site is identified as the glucose transporter. Plasma membranes prepared from diaphragms bind approx. 17 pmol of cytochalasin B/mg of membrane protein to the D-glucose-inhibitable site. If 280 nM (40 000 μunits/ml) insulin is present during incubation, cytochalasin B binding is increased roughly 2-fold without alteration in the dissociation constant of this site. In addition, membranes in the microsomal fraction contain 21 pmol of D-glucose-inhibitable cytochalasin B binding sites/mg of membrane protein. In the presence of insulin during incubation the number of these sites in the microsomal fraction is decreased to 9 pmol/mg of membrane protein. These results suggest that rat diaphragm contain glucose transporters with characteristics identical to those observed for the rat adipose cell glucose transporter. In addition, insulin stimulates glucose transport in rat diaphragm through a translocation of functionally identical glucose transporters from an intracellular membrane pool to the plasma membrane without an alteration in the characteristics of these sites.

Introduction

A primary effect of insulin on muscle and adipose cell function is its stimulation of glucose transport [1–4]. Recently, a unique mechanism has been proposed for the stimulatory action of insulin on glucose transport in adipose cells. Cushman and Wardzala, using a cytochalasin B binding assay for quantitating the number of glucose transporters [5], and Suzuki and Kono, using a reconstitution technique for measuring glucose transport activity [6], have suggested that insulin stimulates glucose transport through a translocation of transporters from a large, membrane-associated intracellular pool to the plasma membrane. More recently, Karnieli et al. [7] have reported that these effects of insulin are rapid, reversible, and insulin concentration-dependent. In addition, Kono et al. [8] have demonstrated that this translocation process and its reversal are energy-dependent, but protein synthesis-independent.

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Muscle plays an important role in systemic glucose homeostasis, probably more so than the adipose cell [9,10]. However, little is known relative to glucose transport in muscle and its regulation by insulin, due primarily to the relatively large size of intact muscle tissue and diffusion-limited access of substrates to the extracellular space. Recently, however, we have reported the quantitation of the number of D-glucose-inhibitable cytochalasin B binding sites in subcellular membrane fractions prepared from rat diaphragm [11]. Using an approach paralleling that reported by Cushman and Wardzala [5] for the adipose cell, we have demonstrated that insulin stimulates glucose transport in the rat diaphragm by a similar translocation of glucose transporters from an intracellular microsomal pool to the plasma membrane.

Previous reports have only eluded to evidence that the D-glucose-inhibitable cytochalasin B binding site represents the glucose transporter in membranes prepared from either adipose cells or muscle [11,24]. These previous reports have not demonstrated that there are similarities between the characteristics of D-glucose-inhibitable cytochalasin B binding and glucose transport itself, except for the specific inhibition of cytochalasin B binding by D-glucose. The present report, therefore, describes in detail the characteristics of this cytochalasin B binding site in rat diaphragm plasma and microsomal membranes which identify this site as the glucose transporter. These studies demonstrate that the parameters for the D-glucose-inhibitable cytochalasin B binding site are comparable to those parameters for the inhibition of glucose transport activity by cytochalasin B. In addition, the present report compares the characteristics of this site before and after insulin stimulation.

Experimental Procedures

For each experiment, 20 ad libitum-fed (standard laboratory chow), 125- to 150-g male rats (albino from a Wistar strain bred in these laboratories) were killed by cervical dislocation and decapitation, and the diaphragms removed intact. The incubation medium was standard Krebs-Ringer bicarbonate buffer reduced to 10 mM HCO_3^-, supplemented with 30 mM Hepes (Sigma Chemical Co.), pH 7.4, and containing 5.6 mM glucose. Following removal, the diaphragms were washed twice in this medium. Ten diaphragms were then placed in each of two flasks containing 25 ml of incubation medium and a final concentration of 0 or 280 nM insulin (Eli Lilly Co., Indianapolis, IN). Incubations were carried out for 30 min at 37°C.

The plasma membrane fraction and a fraction enriched in microsomal membranes were prepared from the incubated diaphragms by a modification of the differential centrifugation method described by Van Alstyne et al. [12]. Five incubated diaphragms were washed in a buffer containing 10 mM $NaHCO_3$ and 5 mM NaN_3, pH 7.0, then initially homogenized in 4 ml of this same buffer at 4°C using a polytron PT 20 homogenizer (Kinematica GmbH, Switzerland) for 60 s at setting 3. The homogenate was centrifuged at $8700 \times g_{max}$ for 20 min. Supernatant-1 was decanted and saved for preparation of the microsomal membrane fraction as described previously [11], and pellet-1 was saved for preparation of the plasma membrane fraction as described by Van Alstyne et al. [12]. Both membrane fractions were ultimately resuspended to a final concentration of 1 to 2 mg of protein/ml in a buffer containing 255 mM sucrose, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4.

Three experiments were also performed to determine the purity of the plasma membrane fraction by layering the crude plasma membrane preparation over a 15 ml linear sucrose gradient (20 to 40%, w/v, verified by refractometry). Following centrifugation of the gradient for 60 min at $135\,000 \times g_{max}$, 20, 750-μl fractions were collected using an ISCO Density Gradient Fractionator (Model 185). Equilibrium D-glucose-inhibitable [^{3}H]cytochalasin B binding to the plasma membrane and microsomal membrane fractions was then measured, and the number of D-glucose-inhibitable binding sites was calculated as previously described for adipose cell plasma membranes and microsomes [5].

In several experiments, glucose transport activity itself was directly measured in the plasma membrane fraction by the gel filtration technique described by Penefsky [13] and Fry et al. [14], with modifications as described by Baldwin et al. [15].

60 μl samples of a plasma membrane vesicle suspension were placed in small tubes in a water bath at 25°C. Hexose uptake was initiated by the addition of 60 μl of a solution of D-[^{14}C]- and L[^{3}H]glucose. The final concentration of radioactivity were about 35 and 70 μCi/ml of ^{14}C and ^{3}H, respectively. At various times, 120-μl aliquots of an ice cold solution containing 10 mM Tris-HCl, 20 μM cytochalasin B, and unlabeled D- and L-glucose at the same concentrations as present in the assay mixture, were added in order to inhibit further uptake.

Plasma membrane vesicles were then separated from the assay mixture as follows. Columns of Sephadex G-50 (fine) were poured to the 1-ml mark in disposable plastic tuberculin syringes. The columns were washed with a solution of 10 mM Tris-HCl, 10 μM cytochalasin B, pH 7.4, containing D- and L-glucose at the same concentrations as present in the assay mixture. Excluded buffer was removed from the columns by centrifugations at 250 × *g* for 3 min. Within 60 s of the completion of hexose uptake 100-μl, aliquots of membrane suspension were applied to the centrifuged columns and allowed to settle into the Sephadex. The syringes were then recentrifuged as described above, with a scintillation vial at the bottom of each centrifuge bucket to collect the eluate. After incubation, all procedures were carried out at 4°C. Uptake of radioactive hexoses were measured by liquid scintillation counting in a Packard Liquid Scintillation Spectrometer.

Protein was determined by the Coomassie brilliant blue method described by Bradford [16] (Bio-Rad protein assay, Bio-Rad Laboratories), using crystalline bovine serum albumin (Sigma Chemical Co.) as the standard. Adenylate cyclase activity was measured as described by Salomon et al. [17] and modified by Cooper et al. [18]. The assay medium contained 0.1 mM ATP, 0.5 μCi of [α-^{32}P]ATP, 4 mM $MgCl_2$, 0.2 mM cyclic AMP, 5 mM creatine phosphate, 25 units/ml of creatine phosphokinase, 30 mM Tris-HCl, pH 7.5, 0.1% bovine serum albumin, and 10 mM NaF. The reaction was initiated by the addition of 2.5 to 5.0 μg of membrane protein to give a total volume of 0.1 ml, and stopped after 10 min at 37°C. Cyclic [^{32}P]AMP was purified as described [17].

Results

The distribution of protein following linear sucrose density gradient centrifugatoin of the crude plasma membrane fraction is illustrated in Fig. 1A. Protein is distributed into two distant peaks, a broad peak at lower sucrose concentrations near the top of the gradient and a narrower peak at higher sucrose concentrations near the bottom of the gradient. Incubation of the diaphragms with insulin did not significantly effect the concentration of protein nor its distribution. Fig. 1B shows, however, that adenylate cyclase activity appears as a single peak corresponding to a small shoulder of protein at low sucrose concentrations (20–22%) *. This peak exhibits a very low UDPgalactose: *N*-acetylglucosamine galactosyltransferase specific activity, a marker enzyme activity characteristic of membranes of the Golgi apparatus [19], relative to that observed in the microsomal membrane fraction (30 and 160 nmol/mg of membrane protein per 2 h, respectively). Illustrated in Fig. 1C, the distribution of D-glucose-inhibitable cytochalasin B binding sites parallels that of adenylate cyclase activity. As also illustrated in Fig. 1C, incubation of intact diaphragms with insulin increases the number of these sites approx. 3-fold. Fractions 6–8 appear, threefore, to represent purified plasma membranes and have been pooled in all further studies reported here.

Fig. 2A illustrates detailed Scatchard plots [20] of equilibrium [^{3}H]cytochalasin B binding at 4°C to purified plasma membranes prepared from intact diaphragms which were incubated for 30 min at 37°C in either the absence or presence of 280 nM (40 000 μunits/ml) insulin. The ratios of bound to free [^{3}H]cytochalasin B represent complex curvilinear functions of the bound cytochalasin B, characteristic of either multiple, noninteracting binding sites, one class of negatively cooperative binding sites, or some combination of the two [21,22]. In the absence of D-glucose, plasma membranes from the insulin-pretreated tissue bind more

* A small but reproducible difference in adenylate cyclase activity has been observed between plasma membranes prepared from basal and insulin-stimulated diaphragms. This effect of insulin is presently under further investigation.

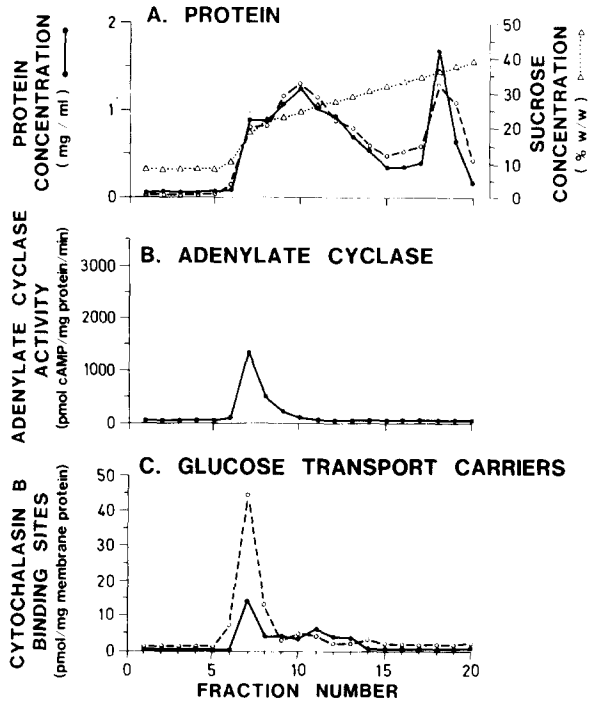

Fig. 1. Distribution of protein, adenylate cyclase activity, and the number of glucose transporters following linear sucrose density gradient centrifugation of a crude plasma membrane fraction from rat diaphragm. A crude plasma membrane fraction was prepared as described under Experimental Procedures and applied to a linear sucrose density gradient, (20–40%, w/v) (△·····△). Subsequent to centrifugation fractions were collected and protein adenylate cyclase activity, and the number of glucose transporters were measured as described. In some preparations, parallel gradients were prepared for measurements of these same parameters of plasma membranes prepared from rat diaphragm incubated in the presence of 280 mM (40000 μunits/ml) insulin (○------○).

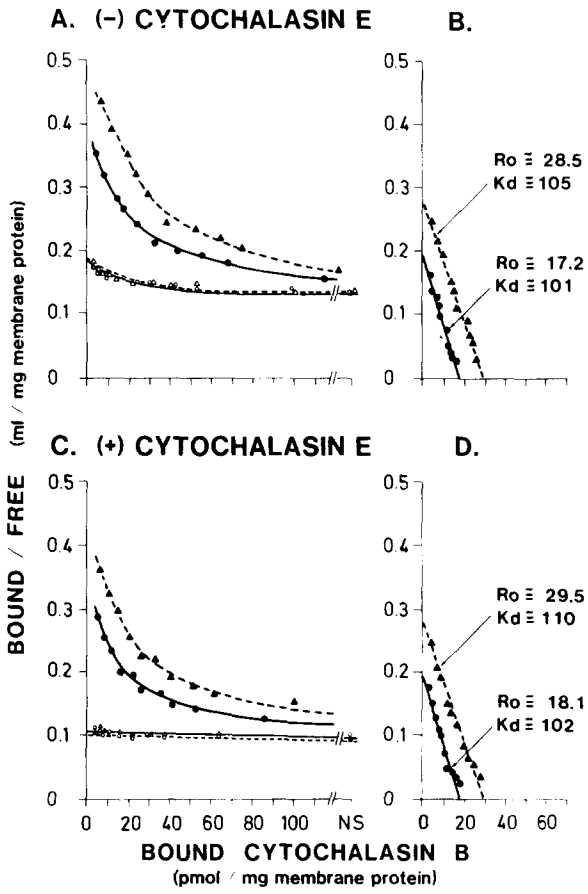

Fig. 2. Scatchard analyses of equilibrium [^{3}H]cytochalasin B binding to isolated rat diaphragm plasma membranes at 4°C. Prior to preparing plasma membranes, intact diaphragms were incubated for 30 min at 37°C in the absence (———) or presence (------) of 280 mM (40000 μunits/ml) insulin. Cytochalasin B binding was then measured over a 1 to 20000 nM range of free cytochalasin B concentrations in the absence (●, ▲) or presence (○, △) of 400 mM D-glucose as described under Experimental Procedures, and the results were plotted (A and C) as described by Scatchard [20]. Derived Scatchard plots (B and D) were constructed by subtracting (along radial axes of constant free cytochalasin B concentrations) each curve obtained in the presence of D-glucose from its respective curve obtained in the absence of D-glucose [21] and analyzing the resulting values by a simple linear regression. Detailed equilibrium [^{3}H]cytochalasin B binding was measured in the absence (A and B) or presence (C and D) of 2000 mM cytochalasin E. (K_d, dissociation constant in nM; R_0, D-glucose-inhibitable cytochalasin B binding sites in pmol/mg of membrane protein).

cytochalasin B than do those from the untreated tissue. 400 mM D-glucose, however, inhibits cytochalasin B binding to both membrane preparations in such a way that the resulting curves are virtually identical.

When these curves are analyzed by subtracting the curves obtained in the presence of 400 mM D-glucose from their respective curves obtained in the absence of D-glucose along radial axes of constant free cytochalasin B concentrations [21], linear Scatchard plots are obtained (Fig. 2B). These linear plots represent the D-glucose-inhibitable class of cytochalasin B binding sites. As shown, insulin increases the number of these binding sites approx. 2-fold from 17.2 to 28.5 pmol/mg of plasma membrane protein without significantly in-

fluencing the K_d of this site (approx. 100 nM).

Cytochalasin E is an analogue of cytochalasin B which does not inhibit either basal or insulin-stimulated glucose transport activity in diaphragm plasma membrane vesicles (Fig. 4). 2000 nM cytochalasin E does, however, slightly inhibit [^{3}H]cytochalasin B binding. Fig. 2C illustrates the effect of cytochalasin E on cytochalasin B binding to plasma membranes. Cytochalasin E reduces cytochalasin B binding primarily by lowering the amount of nonsaturable cytochalasin B binding observed at all cytochalasin B concentrations, but also by inhibiting the small amount of specific binding at low cytochalasin B concentrations not inhibitable by D-glucose. Fig. 2D shows that the presence of 2000 nM cytochalasin E does not alter the characteristics of the D-glucose-inhibitable class of cytochalasin B binding sites.

In order to further characterize the binding of cytochalasin B to diaphragm plasma membranes, the effects of L- and D-glucose on binding were examined in detail. The results are illustrated in Fig. 3. In both the absence and presence of 2000 nM cytochalasin E, the inhibitory effects of 500 mM L-glucose are very small (< 5%) at the cytochalasin B concentration used in these studies (150 nM; a concentration approx. 50% greater than the K_d of the D-glucose-inhibitable cytochalasin B binding site). A similar very small inhibitory effect (< 5%) was observed using plasma membranes from insulin-stimulated diaphragms. On the other hand, D-glucose, increasingly inhibits cytochalasin B binding until saturation of the inhibitory effect is reached at a concentration of roughly 250 mM. The K_i for D-glucose inhibition of cytochalasin B binding is in the range of 25–50 mM. This value is very similar to that determined in human erythrocyte ghosts and plasma membranes [23], as well as adipose cell plasma membranes [4]. In addition, neither insulin nor cytochalasin E significantly effect the K_i for D-glucose inhibition of cytochalasin B binding.

If the D-glucose-inhibitable class of cytochalasin B binding sites represents the glucose transport system, then the dissociation constant (K_d) of these sites for cytochalasin B binding should be comparable to the inhibition constant (K_i) of cytochalasin B for glucose transport activity. Fig. 4 illustrates the concentration-dependent inhibition

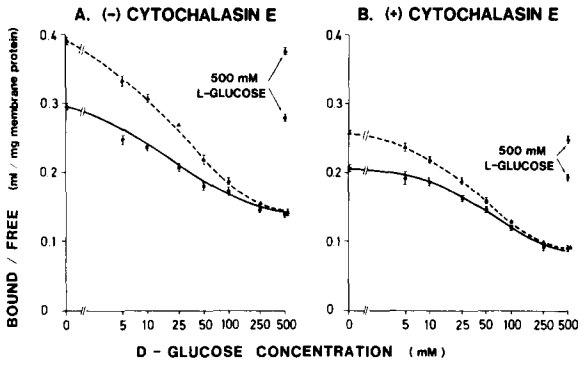

Fig. 3. [^{3}H]Cytochalasin B binding to isolated rat diaphragm plasma membranes at 4°C as a function of D-glucose concentration. Prior to preparing plasma membranes, intact diaphragms were incubated for 30 min at 37°C in the absence (———) or presence (- - - - -) of 280 nM (40000 μunits/ml) insulin. Cytochalasin B binding at a concentration of 150 nM was measured in the absence (A) or presence (B) of 2000 nM cytochalasin E, and in the presence of increasing D-glucose concentrations or 500 mM L-glucose. Results are means ± S.D. of duplicate determinations.

of D-glucose transport by cytochalasin B in diaphragm plasma membrane vesicles at 25°C. While insulin treatment of the intact diaphragms stimulates glucose transport in the plasma membrane

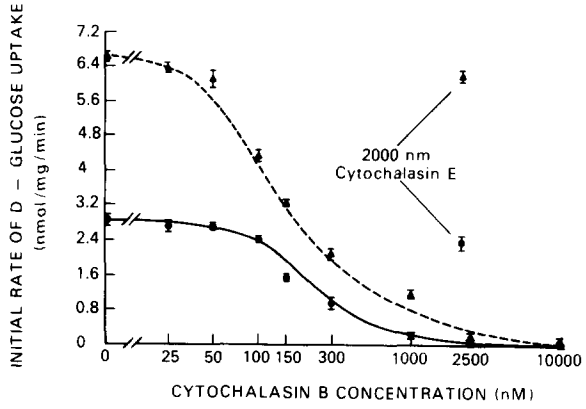

Fig. 4. Glucose transport activity of isolated rat diaphragm plasma membranes at 25°C as a function of cytochalasin B concentration. Prior to preparing plasma membranes, intact diaphragms were incubated for 30 min at 37°C in the absence (———) or presence (- - - - -) of 280 nM (40000 μunits/ml) insulin. Glucose transport activity was measured as described under Experimental Procedures at a concentration of 0.45 mM D-glucose and in the presence of increasing cytochalasin B concentrations. Results are means ± S.D. of duplicate determinations.

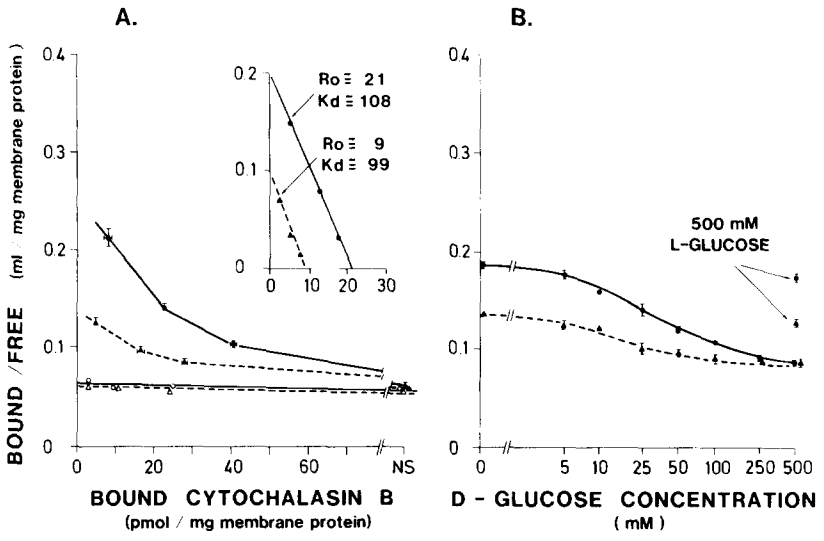

Fig. 5. Scatchard analyses of equilibrium [^{3}H]cytochalasin B binding to isolated rat diaphragm microsomal membrane fractions at 4°C and cytochalasin B binding as a function of D-glucose concentration. Prior to preparing the microsomal membrane fraction, intact diaphragms were incubated for 30 min at 37°C in the absence (———) or presence (- - - - -) of 280 nM (40000 μunits/ml) insulin. All methods were the same as those for plasma membranes in the presence of 2000 nM cytochalasin E in Figs. 2C, 2D and 3B.

vesicles roughly 2-fold, cytochalasin B inhibits glucose transport with a K_i of approx. 150 nM in both membrane preparations. In contrast, and as described above, 2000 nM cytochalasin E inhibits glucose transport only slightly (< 10%).

Fig. 5 summarizes the results of detailed studies of cytochalasin B binding to a rat diaphragm microsomal membrane fraction which, as previously reported, appears to represent the intracellular pool from which glucose transport systems are translocated to the plasma membrane in response to insulin [11]. As shown in Fig. 5A, the D-glucose-inhibitable class of cytochalasin B binding sites in this fraction is characterized by a dissociation constant virtually identical to that observed in the plasma membrane fraction (about 100 nM). While the illustrated studies were performed in the presence of 2000 nM cytochalasin E, similar results are obtained in its absence (data not shown). In contrast to the plasma membranes, however, insulin decreases the number of D-glucose-inhibitable cytochalasin B binding sites in the microsomal membrane fraction from 21 to 9 pmol/mg of membrane protein. In addition, the specific activity of galactosyltransferase is not altered in this microsomal membrane fraction from insulin-stimulated diaphragms as compared to microsomal membranes from basal diaphragms (160 and 155 nmol/mg of membrane protein per 2 h, respectively), indicating that the translocation process is specific for D-glucose-inhibitable cytochalasin B binding sites.

Fig. 5B illustrates the concentration-dependent inhibition of cytochalasin B binding by D-glucose in these same microsomal membrane preparations. Inhibition curves similar to those observed with plasma membranes obtained whether microsomes were prepared from basal or insulin-stimulated diaphragms. Under all conditions examined (± cytochalasin E, ± insulin), the K_i for D-glucose inhibition is roughly 25–50 mM.

Discussion

Using a D-glucose-inhibitable [^{3}H]cytochalasin B binding assay to quantitate the number of glucose transporters in subcellular membrane fractions, a previous report from this laboratory demonstrated that insulin appears to stimulate glucose transport in rat diaphragm through a translocation of glucose transporters from an intracellular membrane pool to the plasma membrane. The studies

reported here provide strong evidence that the D-glucose-inhibitable class of cytochalasin B binding sites in rat diaphragm plasma and microsomal membranes represents the glucose transporter and that the characteristics of this site are very similar to those reported for the human erythrocyte [25,26] and rat adipose cell [24].

A direct identification of the D-glucose-inhibitable cytochalasin B binding site as the glucose transporter has been possible in human erythrocyte ghosts and plasma membranes and in rat adipose cell plasma membranes since both cytochalasin B binding and glucose transport activity can be measured in these preparations. As shown in the present study, a similar direct identification can be made in rat diaphragm plasma membranes. Scatchard analysis of detailed equilibrium [^{3}H]cytochalasin B binding curves in the absence and presence of a saturating concentration of D-glucose demonstrate a single class of D-glucose-inhibitable binding sites (Fig. 2A and B). The dissociation constant (K_d) of this binding site (approx. 100 nM) is very similar to that observed in both human erythrocyte ghosts and plasma membranes [25,26] and rat adipose cell plasma membranes [24]. Second, the inhibition of binding to this site is specific for D-glucose (Fig. 3). D-Glucose, in a concentration-dependent manner, inhibits cytochalasin B binding to this site, whereas L-glucose has only a small, insignificant effect. The K_i for D-glucose inhibition of cytochalasin B binding is roughly 25–50 mM, a range similar to that reported for both the human erythrocyte [23] and rat adipose cell [24].

Third, cytochalasin B inhibits glucose transport activity itself in rat diaphragm plasma membrane vesicles with a K_i of approx. 150 nM, a value quite close that for the K_d of the D-glucose-inhibitable binding site for cytochalasin B binding. Finally, cytochalasin E a cytochalasin B analogue which does not inhibit glucose transport activity in rat diaphragm plasma membrane vesicles (Fig. 4), has but a small inhibitory effect on total cytochalasin B binding and none at all on binding to the D-glucose-inhibitable site (Figs. 2 and 3). This failure of cytochalasin E to alter the characteristics of cytochalasin B binding to the D-glucose-inhibitable site in rat diaphragm plasma membranes parallels that observed in rat adipose cell plasma membranes where a large effect of cytochalasin E is observed on binding to sites other than the D-glucose-inhibitable site [24].

More similar attempts to measure glucose uptake into microsomal membranes prepared from diaphragm, however, have been unsuccessful and further characterization is being performed. But, based on the characterization of the glucose transporters in adipose cell microsomal membranes, by Cushman and Wardzala [5] and Suzuki and Kono [6], identification of the D-glucose-inhibitable cytochalasin B binding site in diaphragm microsomal membranes as the glucose transporters seems likely.

The studies reported here further demonstrate that the apparent translocation of glucose transporters from the microsomal membrane pool to the plasma membrane induced by insulin in rat diaphragm is not accompanied by significant alterations in the characteristics of the D-glucose-inhibitable cytochalasin B binding site. Insulin does not influence either the K_d of this site for cytochalasin B binding (Figs. 2 and 5) or the K_i of D-glucose for cytochalasin B binding to this site in both the plasma and microsomal membrane fractions, or the K_i of cytochalasin B for glucose transport itself in plasma membrane vesicles (Fig. 4). Preliminary studies also indicate that insulin induces this apparent translocation of glucose transport systems in a concentration-dependent manner with a half maximal effect at 1.4 nM (200 μU/ml) and a maximal effect at 3.5 nM (500 μU/ml) (data not shown). Similar values are observed when glucose uptake is examined in the intact rat diaphragm prior to preparation of the membrane fractions.

Recently, this laboratory has presented data demonstrating that a D-glucose inhibitable cytochalasin B binding can be measured to plasma and microsomal membranes prepared from rat diaphragm [11]. The method for preparing plasma membranes was based on the method of Boegman et al. [27] and involved a high salt extraction. This method was long and at times not very reproducible. In this present study, a much faster and more reproducible preparation is used as has been described by Van Alstyne et al. [12]. The membranes prepared with this preparation have high adenylate cyclase activity and a high level of the number of D-glucose-inhibitable cytochalasin B binding

sites (Fig. 1B). Both of these activities are very similar in degree of those measured using the high-salt extraction method as described previously. The present method is currently being used due to the ease and reproducible nature of the preparation. The characterization of [^{3}H]cytochalasin B binding to these plasma membranes and measurements of glucose transport activity in these membranes have demonstrated that a D-glucose inhibitable cytochalasin B binding site can be identified as the glucose transporter. Furthermore, exposure of intact rat diaphragms to insulin for 30 min at 37°C appears to bring about a translocation of apparently identical glucose transporters from an intracellular membrane pool to the plasma membrane. The consequence of such a translocation is a 2-fold increase in the number of transporters in the plasma membrane. Preliminary studies also indicate that this effect of insulin is concentration-dependent and correlates well with the effect of insulin on glucose uptake in the intact diaphragm. These results show that the characteristics of the glucose transporter in rat diaphragm is very similar to the carrier of rat adipose cells [24] and the erythrocyte [25]. Finally, these results give further support to the mechanism of the acute action of insulin on glucose transport as proposed by Cushman and Wardzala [5] and Suzuki and Kono [6] in rat adipose cells and indicate that insulin may stimulate glucose transport in insulin-sensitive cell types through a comparable mechanism.

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