0026-895X/91/030383-07$03.00/0

MOLECULAR PHARMACOLOGY, 40:383-389

Differentiation of Erythrocyte- (GLUT1), Liver- (GLUT2), and Adipocyte-Type (GLUT4) Glucose Transporters by Binding of the Inhibitory Ligands Cytochalasin B, Forskolin, Dipyridamole, and Isobutylmethylxanthine

BETTINA HELLWIG and HANS G. JOOST

Institut für Pharmakologie und Toxikologie der Universität Göttingen, D-3400 Göttingen, FRG

Received January 9, 1991; Accepted June 12, 1991

SUMMARY

The binding affinities of the glucose transporter isoforms GLUT1, GLUT2, and GLUT4 for the inhibitory ligands cytochalasin B, forskolin, dipyridamole, and isobutylmethylxanthine (IBMX) were compared in membranes from human erythrocytes and rat brain containing the erythrocyte-type glucose transporter (GLUT1), in membranes from rat liver containing the liver-type glucose transporter (GLUT2), and in membranes from adipocytes and heart containing predominantly the adipose/muscle-type glucose transporter (GLUT4). The binding affinities of cytochalasin B for GLUT1 and GLUT4 were virtually identical (K_D) in membranes from erythrocytes, 190 nM; in brain, 130 nM; in adipocytes, 160 nM; and in heart, 170 nM). In contrast, no specific glucose-inhibitable binding of cytochalasin B was detected in liver membranes. The binding affinity for forskolin of GLUT1 was significantly lower than that of GLUT4 (K_D in erythrocytes, 2360 nM; K_I in brain, 4360 nM; and K_D in adipocytes, 200 nM; and in heart, 210 nM); specific glucose-inhibitable binding to GLUT2 was not detectable. Like forskolin, the glucose transport inhibitors dipyridamole (K_I in adipocyte membranes, 1.2 μM; in erythrocytes, >40 μM) and IBMX (K_I in adipocyte membranes, 60 μM; and in erythrocytes, >500 μM) bound with higher affinity to GLUT4 than to GLUT1. These data demonstrate striking differences of GLUT1, GLUT2, and GLUT4 with respect to their binding affinity for the inhibitory ligands cytochalasin B, forskolin, dipyridamole, and IBMX. It is suggested that the complex differences result from interaction of more than one heterogeneous binding site at the glucose transporters with the inhibitory ligand.

The diterpene forskolin (1, 2) is a powerful inhibitor of the carrier-mediated diffusion of D-glucose into adipocytes and erythrocytes (3, 4). Several lines of evidence have indicated that this inhibitory effect is mediated by a direct interaction of the diterpene with the glucose transporter, rather than by its stimulatory effect on adenylate cyclase activity. Firstly, in adipocytes prostaglandin E_2 inhibited the effect of forskolin on lipolysis but failed to reverse the inhibition of glucose transport (5). Secondly, the concentrations of forskolin inhibiting glucose transport in adipocytes were approximately 1 order of magnitude lower than those necessary for stimulation of adenylate cyclase (6). Thirdly, the forskolin derivative 1,9-dideoxy-forskolin inhibited glucose transport but failed to stimulate adenylate cyclase (6). It was concluded from these data that glucose transporters possess a binding site that, upon occupation by forskolin or other inhibitory ligands, mediates a competitive inhibition of glucose transport. Indeed, forskolin inhibited the binding of cytochalasin B to glucose transporters in membranes from adipocytes (5), erythrocytes (4), and platelets (7), in a competitive manner. Furthermore, glucose transporters in erythrocyte membranes were covalently labeled by photolysis in the presence of [^{3}H]forskolin (8) or of an iodinated, photoreactive, forskolin derivative, IAPS-forskolin (9).

Glucose transporters represent a family of structurally similar membrane proteins encoded by different genes. These transporters differ considerably in their tissue distribution (for a recent review, see Ref. 10); GLUT1 is mainly expressed by brain and erythrocytes and GLUT2 by liver and pancreatic β cells, and GLUT4 is exclusively expressed in tissues equipped with an insulin-sensitive glucose transport, namely, muscle and adipose tissue. The striking heterogeneity in structure and tissue distribution of the glucose transporters raises the question of functional differences between the transporter species. Therefore, we have compared the binding affinities of the glucose transporter species GLUT1, GLUT2, and GLUT4 for the inhibitory ligands forskolin and cytochalasin B. Because binding of these ligands to glucose transporters is competitively inhibited by D-glucose, it is assumed to reflect an interaction

This study has been supported by the Deutsche Forschungsgemeinschaft. B.H. is the recipient of a fellowship from the Friedrich-Naumann-Stiftung.

ABBREVIATIONS: IAPS-forskolin, 3-Iodo-4azidophenethylamido-7-0-succinyldeacetylforskolin; IBMX, isobutylmethylxanthine.

with the catalytic site of the protein responsible for the transport of the hexose. Thus, we anticipated that functional differences between the transporter species would become apparent in the binding characteristics of the inhibitory ligands. In addition, we included IBMX and dipyridamole in the study, two inhibitors of glucose transport that have previously been shown to bind to the glucose transporter in adipocytes (11, 12). The data indicate that the erythrocyte-type glucose transporter (GLUT1) differs from the adipocyte/muscle-type glucose transporter (GLUT4) in its considerably lower affinity for forskolin and the transport inhibitors dipyridamole and IBMX, but not in its affinity for cytochalasin B. The affinity for cytochalasin B and forskolin of the liver-type glucose transporter (GLUT2), in contrast, was below the level of detectability.

Experimental Procedures

Materials. Unlabeled forskolin was a generous gift from Dr. H. Metzger, Hoechst AG (Frankfurt, FRG). [^{3}H]Forskolin was purchased from Amersham/Buchler (Braunschweig, FRG). [^{3}H]Cytochalasin B was from New England Nuclear (Dreieich, FRG).

Preparation of plasma membranes. Erythrocyte ghosts were prepared from freshly drawn human blood, as described previously (13). Plasma membranes from insulin-treated rat adipocytes were prepared according to a previously published procedure (14, 15). Crude brain membranes were prepared from two to four rats by homogenization of total brains, devoid of cerebellum, with a Potter Elvehjem homogenizer (no. 3431-E25; A. H. Thomas, Philadelphia, PA) (15 strokes at 500 rpm), in 30–60 ml (15 ml/rat) of a 0.32 M sucrose solution. After centrifugation of the homogenate at 1,000 × *g* for 10 min, the supernatant was centrifuged for 30 min at 45,000 × *g*. The resulting pellet was resuspended in Tris buffer (10 mM, pH 7.4) and centrifuged again. The washing step was repeated, and the final pellet was resuspended in the same buffer to a protein concentration of approximately 10 mg/ml.

A crude liver membrane preparation was obtained by homogenization of 10 g of rat liver, with a Potter Elvehjem homogenizer (10 strokes at 500 rpm), in Tris-buffered sucrose (10 mM Tris, 0.32 M sucrose, pH 7.4). After centrifugation of the homogenate at 800 × *g* for 10 min, the supernatant was centrifuged for 25 min at 45,000 × *g*. The resulting pellet was layered onto a cushion of 38% sucrose and was centrifuged at 100,000 × *g* for 60 min. Membranes were collected from the interface, washed once with 10 mM Tris buffer (pH 7.4), and resuspended in the same buffer to a protein concentration of approximately 3 mg/ml.

Several different procedures were tested for the preparation of a heart membrane preparation enriched in glucose transporters. As judged from the immunoreactivity of GLUT4 in the preparations, a modification of the procedure employed for preparation of adipocyte membranes yielded the best results. Hearts were dissected from five rats, suspended in 0.25 M sucrose solution, minced with scissors, and homogenized for 2 × 30 sec with a Polytron homogenizer (Ultraturrax). The homogenate was centrifuged at 600 × *g* for 10 min, and the resulting supernatant was centrifuged again at 100,000 × *g* for 30 min. The pellet of the second centrifugation was resuspended in Tris-EDTA-sucrose buffer, layered on a 38% sucrose cushion, and centrifuged for 60 min at 100,000 × *g*. Membranes were collected from the top of the sucrose layer, washed once with Tris buffer (10 mM, pH 7.4), and resuspended in the same buffer at a protein concentration of approximately 2 mg/ml.

Immunoblotting of glucose transporters. Antiserum against the carboxyl terminus of the rat/human GLUT1 [peptide in one-letter code, (C)EELFHPLGADSQV (16)] was a gift from Dr. S. Cushman (National Institutes of Health, Bethesda, MD). Antiserum against the rat muscle/adipose tissue glucose transporter (GLUT4) was raised with a peptide corresponding to the carboxyl terminus of this transporter (sequence in one-letter code, STELEYLGPDEND) coupled to keyhole limpet hemocyanin. Antiserum against a peptide corresponding to the carboxyl terminus of the rat liver-type glucose transporter (GLUT2) was a gift from Dr. B. Thorens (Whitehead Institute, Cambridge, MA) (17). Samples of the membrane fractions (50 μg of protein from adipocyte, heart muscle, and brain membranes; 5 μg from erythrocyte membranes) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose sheets (Schleicher & Schuell, Dassel, FRG) with a semi-dry blotting apparatus (Sartoblot II; Sartorius, Göttingen, FRG). Sheets were blocked overnight by incubation with buffer containing 0.05% (w/v) Tween 20 and 150 mM sodium chloride and were incubated with the antiserum, diluted in blocking buffer at 1/200, for 2 hr at room temperature. After three washes with buffer containing albumin (1%), sheets were incubated with ^{125}I-Protein A (0.1 μCi/ml) for 1 hr, thoroughly washed, and autoradiographed for 1–4 days.

Binding assays. Samples of membrane protein (approximately 50 μg) were incubated with tritiated forskolin or cytochalasin B (0.05 μCi in the experiments with erythrocyte membranes; 0.2 μCi for all other membranes), [^{14}C]urea, cytochalasin E (14), and the indicated concentrations of unlabeled agents, for 10 min on ice. Samples containing 200 mM D-glucose were run in parallel, in order to determine the portion of total binding unrelated to glucose transporters (nonspecific binding). The amount of nonspecific binding assayed with D-glucose corresponded to that assayed with unlabeled forskolin when tracer cytochalasin B was used or with cytochalasin B when tracer forskolin was employed. Membranes were separated from the buffer by centrifugation for 30 min in a refrigerated microfuge at 15,000 rpm (24,000 × *g*). This centrifugation was sufficient to pellet all plasma membranes. After removal of the incubation buffer, tips of the tubes containing the pellets were cut off and transferred into scintillation vials, and 0.5 ml of a tissue solubilizer (TS1; Zinsser Analytic, Frankfurt, FRG) was added. The samples were shaken for 120 min, and a toluene-based scintillator was added for determination of the radioactivity incorporated into the membrane pellet.

Calculations. The binding curves were fitted with the IBM-PC version of the program GraphPad. This computerized procedure included a correction of the binding data for a low affinity component of the total binding that was not inhibitable by glucose. The procedure yielded essentially the same results as the previously employed graphic evaluation of binding curves assayed in the presence or absence of glucose (14). Means ± standard errors of the resulting dissociation constants and numbers of sites derived from several independent experiments were calculated and are presented in Table 1. K_I values were calculated according to the method of Cheng and Prusoff (18). In order to facilitate the comparison of different membrane preparations, binding data, as presented in Figs. 2–6, were normalized to percentage of maximal binding.

Results

Immunochemical characterization of the membrane preparations from erythrocytes, brain, liver, adipocytes, and heart, with respect to their content of GLUT1, GLUT2, and GLUT4. In order to identify the glucose transporters in the membrane preparations used in the binding assays, we employed antisera raised against peptides corresponding to the carboxyl-terminal sequences of the erythrocyte-type (GLUT1), the liver-type (GLUT2), and the adipose/muscle-type (GLUT4) glucose transporters. Immunoblots (Fig. 1) illustrate that erythrocyte and brain membranes contained the erythrocyte-type glucose transporter (GLUT1) and were devoid of GLUT2 and GLUT4. GLUT1 was much more abundant in erythrocytes than in brain, inasmuch as 50 μg of brain membranes yielded a weaker signal than that produced by 5 μg of erythrocyte membrane protein (Fig. 1). Even after a prolonged exposure of the immunoblot (not shown), the adipose/muscle-

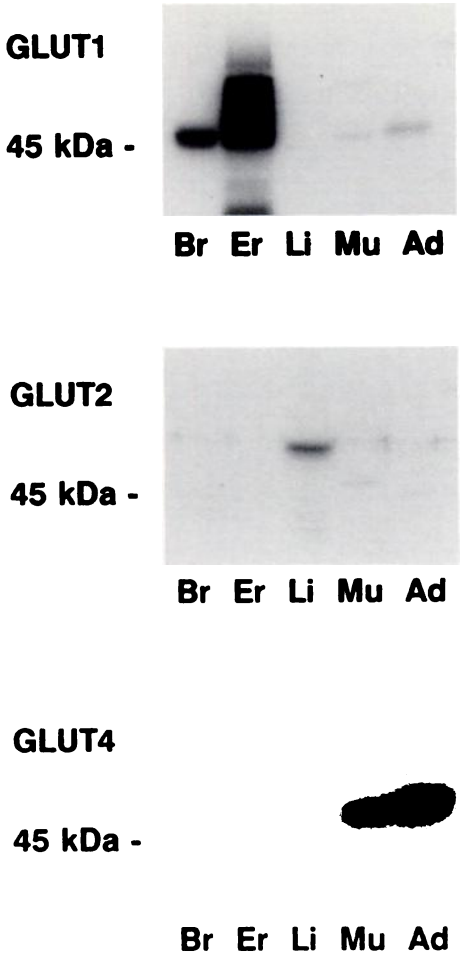

Fig. 1. Immunochemical detection of the glucose transporters GLUT1 and GLUT4 in membranes from brain (*Br*), erythrocytes (*Er*), liver (*Li*), heart muscle (*Mu*), and adipocytes (*Ad*). Samples of 5 (erythrocytes) or 50 μg (all other samples) of membrane protein were separated by electrophoresis and transferred onto nitrocellulose membranes. The blots were immunoassayed, as described, with anti-GLUT1 (*top*), anti-GLUT2 (*middle*), or ant-GLUT4 (*bottom*) antiserum.

type transporter (GLUT4) was undetectable in membrane from brain or erythrocytes.

Liver membranes contained exclusively the GLUT2, whereas the membranes from adipocytes and heart muscle contained predominantly GLUT4 (19), contained minute amounts of GLUT1, and were devoid of GLUT2 (Fig. 1). The amount of GLUT1 present in adipocytes and heart muscle can be estimated on the basis of the relative immunoreactivities and the number of cytochalasin B binding sites; the immunoreactivity of GLUT1 in adipocyte and heart membranes was approximately 10-fold lower than that in brain membranes. Based on the assumption that the immunoreactivity of GLUT1 in brain membranes reflects 16 pmol of glucose transporters/mg of membrane protein (cytochalasin B binding sites, from Table 1), GLUT1 in adipocyte and heart membranes would amount to about 1.6 pmol/mg and would, therefore, represent approximately 5–10% of the total glucose transporters (cytochalasin B binding sites).

Binding of forskolin to glucose transporters in membrane from erythrocytes, adipocytes, and heart. Binding of forskolin to glucose transporters in membranes from adipocytes, heart, and erythrocytes was assayed by displacement of [^{3}H]forskolin with unlabeled forskolin. A large portion of the total binding of forskolin was fully inhibited by both glucose and cytochalasin B and was, therefore, due to binding of the diterpene to the glucose transporter. After correction of the data for the nonspecific component, a single K_D of forskolin binding resulted. As illustrated in Fig. 2, the binding curves of [^{3}H]forskolin binding in membranes from adipocytes and heart were virtually superimposable. In erythrocyte membranes, in contrast, higher concentrations of forskolin were necessary to inhibit binding of the tracer. Accordingly, the K_D of forskolin binding deduced from several experiments (Table 1) was 1 order of magnitude higher in erythrocyte membranes than in heart or adipocyte membranes.

Fig. 3 illustrates the concentration-dependent inhibition of binding of [^{3}H]forskolin by unlabeled cytochalasin B in erythrocyte, heart, and adipocyte membranes. In contrast to the K_D values of forskolin (Fig. 2; Table 1), the K_I values of cytochalasin B, as determined by inhibition of [^{3}H]forskolin binding with cytochalasin B, were almost identical in these membrane preparations (Fig. 3; Table 1). Thus, the data show a striking difference in the binding affinities of glucose transporters GLUT1 and GLUT4 for forskolin, whereas their binding affinities for cytochalasin B appeared identical.

Based on the data obtained with [^{3}H]forskolin in erythrocyte membranes and with [^{3}H]cytochalasin B, we expected a K_D of > 2 μM for the glucose-inhibitable forskolin binding to the GLUT1 in brain membranes. Binding curves of tritiated forskolin to brain membranes revealed a high affinity binding site (K_D of about 100 nM), which was not inhibited by glucose or cytochalasin B (data not shown). This site probably represents the previously described binding site of forskolin at the catalytic subunit of adenylate cyclase (20). The presence of this high affinity binding site unrelated to the glucose transporter interfered with the detection of glucose-inhibitable [^{3}H]forskolin binding, because of the relatively low abundance of the GLUT1 and its low affinity for forskolin in brain membranes. Only 10% of the total binding was inhibited by glucose; thus, we were unable to get a reliable result for the K_D of the glucose-inhibitable [^{3}H]forskolin binding in brain membranes. Erythrocyte membranes, in contrast, contain approximately 10 times more glucose transporters than do brain membranes and lack the high affinity binding site for forskolin that is unrelated to the glucose transporter.

Binding of cytochalasin B to glucose transporters in membranes from erythrocytes, brain, adipocytes, and heart. Glucose-inhibitable [^{3}H]cytochalasin B binding was determined, in order to ascertain that the cytochalasin B binding sites had a similar affinity in brain, erythrocytes, adipocytes, and heart. Indeed, as is shown in Table 1, the K_D values from the respective membrane preparations were virtually identical. It should be noted that in adipocyte and muscle membranes the K_D values of [^{3}H]cytochalasin B binding appeared to be higher than the corresponding K_I values of cytochalasin B in inhibiting [^{3}H]forskolin binding (Table 1).

Fig. 4 illustrates the concentration-dependent inhibition of binding of [^{3}H]cytochalasin B by forskolin in membranes from erythrocytes, brain, adipocytes, and heart. The inhibitory potency of forskolin appeared identical in adipocyte and heart membranes. In erythrocyte membranes, in contrast, half-maximally inhibitory concentrations were about 1 order of magni-

TABLE 1

Binding characteristics of forskolin and cytochalasin B to glucose transporters in membranes from human erythrocytes and rat adipocytes, brain, and heart

Preparation of membranes and binding assays were carried out as described in Experimental Procedures. Adipocyte membranes were from insulin-treated fat cells. K_D values were determined by a computerized fit and correction for the nonspecific binding not inhibitable by D-glucose. K_I values were calculated from IC_{50} values as described (18).

	Erythrocyte	Brain	Adipocyte	Cardiac muscle
[^{3}H]Forskolin binding				
K_D (nM)	2360 ± 690	ND*	200 ± 30	210 ± 30
K_I of cytochalasin (nM)	170 ± 30	ND	90 ± 20	80 ± 10
Number of sites (pmol/mg)	290 ± 54	ND	13 ± 4	11 ± 5
[^{3}H]Cytochalasin B binding				
K_D (nM)	190 ± 50	130 ± 30	160 ± 50	170 ± 30
K_I of forskolin (nM)	2220 ± 660	4400 ± 1700	410 ± 90	370 ± 110
Number of sites (pmol/mg)	230 ± 40	16 ± 4	25 ± 6	15 ± 3

* ND, not detected.

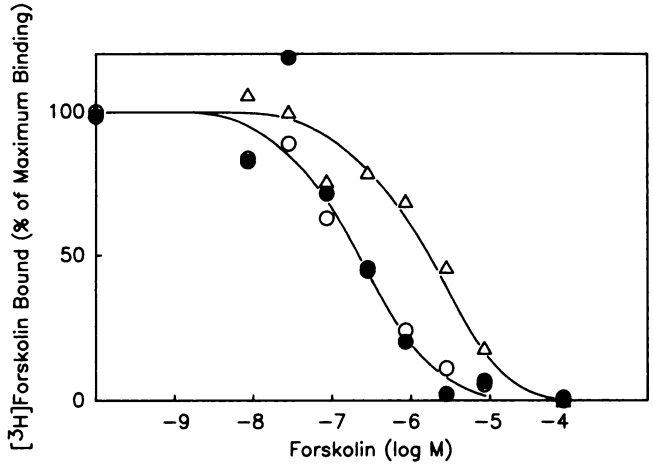

Fig. 2. Concentration-dependent inhibition of [^{3}H]forskolin binding by unlabeled forskolin in membranes from erythrocytes (△), adipocytes (○), and heart (●). Binding assays were performed on samples of 15 (erythrocytes) or 40 μg of membrane protein, as described in Experimental Procedures. The data were corrected for nonspecific binding and were normalized for the maximum binding in each membrane preparation.

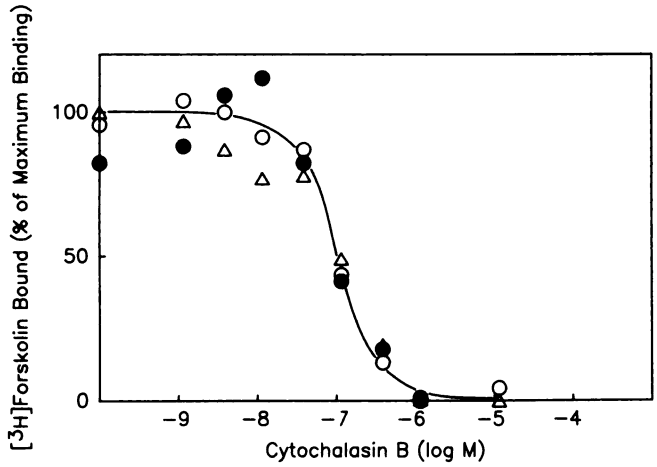

Fig. 3. Concentration-dependent inhibition of [^{3}H]forskolin binding by cytochalasin B in membranes from erythrocytes (△), adipocytes (○), and heart (●). Binding assays were performed on samples of 15 (erythrocytes) or 40 μg of membrane protein, as described in Experimental Procedures. The data were corrected for nonspecific binding and were normalized for the maximum binding in each membrane preparation.

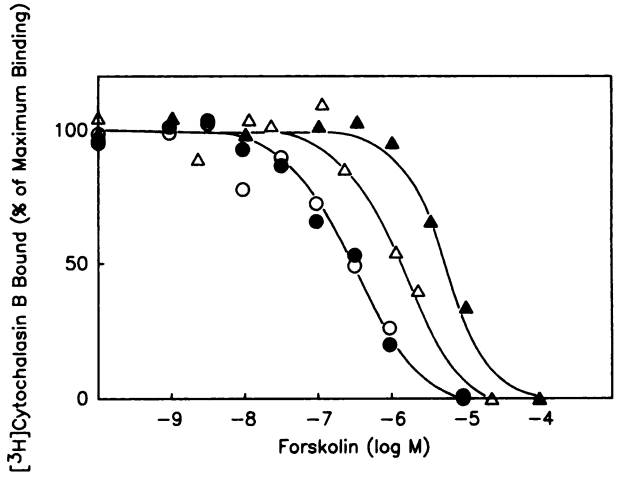

Fig. 4. Concentration-dependent inhibition of [^{3}H]cytochalasin B binding by forskolin in membranes from erythrocytes (△), brain (▲), adipocytes (○), and heart (●). Binding assays were performed on samples of 15 (erythrocytes) or 40 μg of membrane protein, as described in Experimental Procedures. The data were corrected for nonspecific binding and were normalized for the maximum binding in each membrane preparation.

tude higher than in membranes from adipocytes and heart (Fig. 4; Table 1). In brain membranes, the K_I of forskolin was even higher than in erythrocyte membranes (Fig. 4; Table 1). Again, it should be noted that the K_I values of forskolin in inhibiting [^{3}H]cytochalasin B binding in heart adipocytes were higher than the corresponding K_D values of [^{3}H]forskolin binding (Table 1).

Binding of forskolin and cytochalasin B to membranes from rat liver. The binding of [^{3}H]cytochalasin B (K_D = 5 μM) and [^{3}H]forskolin (K_D = 0.15 μM) in liver membranes was specifically inhibited by cytochalasin B and forskolin, respectively (Fig. 5). However, these binding sites were unrelated to the glucose transporter, inasmuch as binding of neither agent was significantly inhibited by D-glucose. Thus, we failed to detect a specific binding site for the inhibitory ligands at the GLUT2.

Inhibition of binding of cytochalasin B by the transport inhibitors dipyridamole and IBMX in membranes from adipocytes and erythrocytes. We have previously shown that the pyrimidine derivative dipyridamole inhibits glucose-displaceable cytochalasin B binding in adipocyte membranes (12), and we have concluded that this inhibitory effect reflects the binding of the agent to a site at the glucose trans-

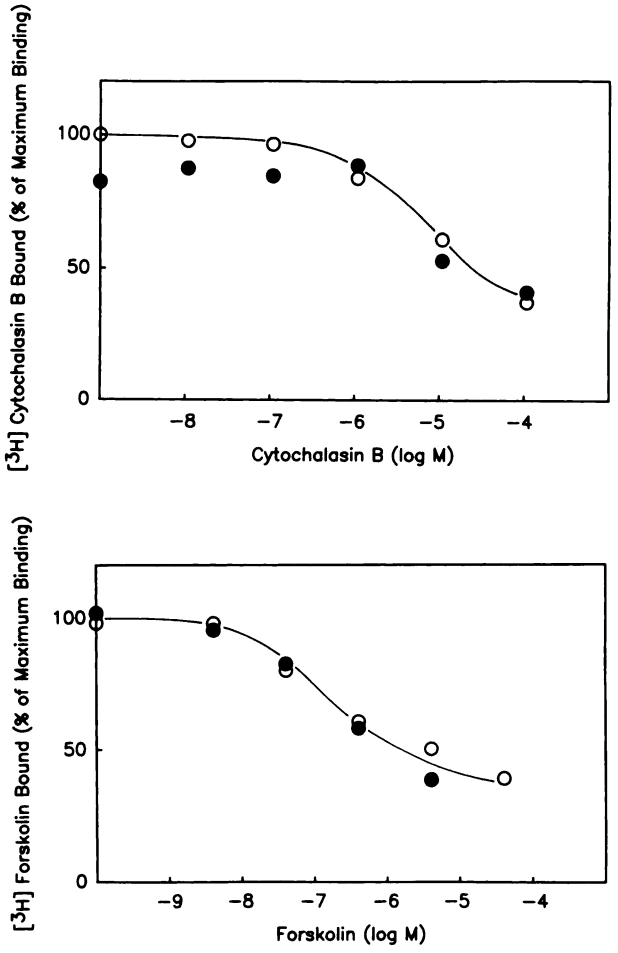

Fig. 5. Binding of cytochalasin B (*upper*) and forskolin (*lower*) to liver membranes. Binding assays were performed on samples of 100 μg of membrane protein, as described in Experimental Procedures, in the presence (●) or absence (○) of 200 mM D-glucose. The data were normalized for maximum binding in each membrane preparation.

porter. As illustrated in Fig. 6, dipyridamole was considerably more potent in inhibiting cytochalasin B binding in adipocyte membranes than in erythrocyte membranes (K_I in adipocyte membranes, 1.2 ± 0.25 μM; in erythrocyte membranes, >40 μM). Like dipyridamole, the methylxanthine IBMX appears to inhibit glucose transport by a direct interaction with the glucose transporter (11). Accordingly, IBMX inhibited the glucose-displaceable [^{3}H]cytochalasin binding in membranes from adipocytes (Fig. 7) with a K_I of 61 ± 20 μM, similar to its half-maximally inhibitory concentration in intact adipocytes (100 μM) (11). In contrast, as is illustrated in Fig. 7, IBMX was much less potent in inhibiting [^{3}H]cytochalasin B binding in membranes from erythrocytes (K_D of >500 μM).

Discussion

The discovery of different types of glucose transporters with a high degree of tissue specificity (19) raises the question as to whether specific functional characteristics are determined by the structural differences. It is obvious that a tissue-specific heterogeneity of glucose transporters might allow a selective regulation of the transport activity according to the demand of individual organs. The data of the present study indicate that the glucose transporters GLUT1, GLUT2, and GLUT4 differ significantly in their affinity for transport-inhibiting ligands, e.g., cytochalasin B, forskolin, IBMX, and dipyridamole. GLUT1 and GLUT4 bind cytochalasin B with identical and high affinity, whereas GLUT2 lacks this high affinity binding site. Furthermore, GLUT1 and GLUT4 differ considerably in their affinity for forskolin, dipyridamole, and IBMX. Because of the competitive interaction of its ligands with glucose, the cytochalasin B binding site is believed to represent the site of the glucose transporter that interacts with the transported hexose. Thus, identification of the structural elements responsible for the differences in binding of ligands to the glucose transporters GLUT1, GLUT2, and GLUT4 will provide information on the specific domains of these transporters responsible for functional differences.

The results of the present study reconcile some discrepancies that become apparent from a comparison of previous reports.

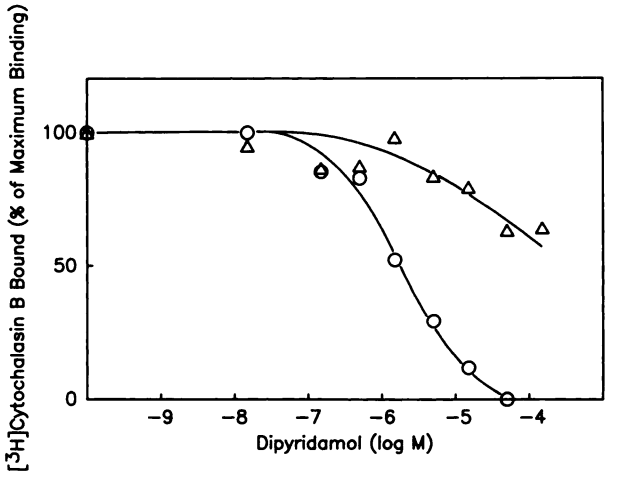

Fig. 6. Concentration-dependent inhibition of [^{3}H]cytochalasin B binding by dipyridamole in membranes from erythrocytes (△) and adipocytes (○). Binding assays were performed on samples of 15 (erythrocytes) or 40 μg of membrane protein, as described in Experimental Procedures. The data were corrected for nonspecific binding and were normalized for the maximum binding in each membrane preparation.

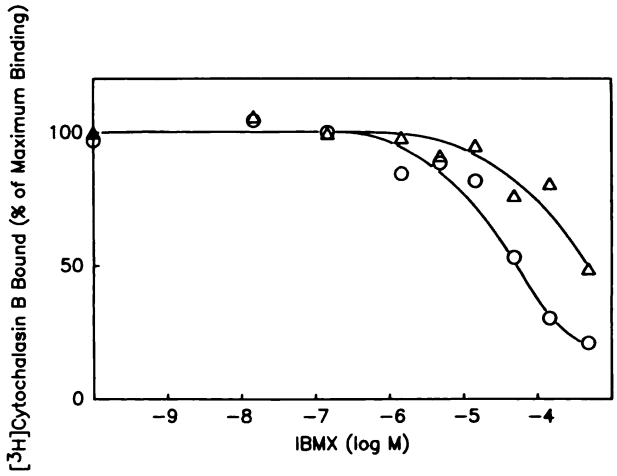

Fig. 7. Concentration-dependent inhibition of [^{3}H]cytochalasin B binding by IBMX in membranes from erythrocytes (△) and adipocytes (○). Binding assays were performed on samples of 15 (erythrocytes) or 40 μg of membrane protein, as described in Experimental Procedures. The data were corrected for nonspecific binding and were normalized for the maximum binding in each membrane preparation.

In erythrocyte membranes, micromolar concentrations of forskolin were necessary to inhibit glucose transport and [^{3}H] cytochalasin B binding (4). In a more recent study (5), in contrast, the concentrations of forskolin inhibiting glucose transport and cytochalasin B binding in adipocyte membranes were reported to be about 1 order of magnitude lower than in the previous study (4). Furthermore, the K_D of [^{3}H]forskolin in erythrocyte membranes was considerably higher than that of cytochalasin B (20), whereas in plasma membranes from insulin-treated adipocytes similar concentrations of forskolin and cytochalasin B inhibited [^{3}H]cytochalasin B binding and glucose transport (5, 6). The present data indicate that these discrepancies reflect complex differences in the ligand binding sites of the glucose transporters GLUT1 and GLUT4.

Binding of cytochalasin B to liver membranes has been investigated in two previous studies (21, 22). In both studies, glucose-inhibitable binding of cytochalasin B was detected, albeit of very low affinity (K_D = 1.7 μM) (21). The failure of the present study to detect any glucose-inhibitable cytochalasin B binding in liver membranes does not necessarily contradict these reports, because it may be very difficult to detect a low affinity binding site against a background of other sites. Given that the K_D of cytochalasin B binding to the GLUT2 in our liver membranes is lower than 2 μM and that the total number of sites is 40 pmol/mg (22), the glucose-inhibitable binding in our assay would be <15% of the total binding. Thus, our assay might not be sensitive enough to detect the low affinity binding site described previously. It appears reasonable to conclude, however, that GLUT2 lacks a high affinity binding site for cytochalasin B and forskolin.

It has recently been reported that glucose transport in L6 muscle cells is more sensitive to inhibition by forskolin after treatment with insulin than in the basal state (23). The present findings provide a reasonable explanation for this difference, on the basis of the assumption that GLUT1 is the predominant glucose transporter in plasma membranes of basal cells, whereas it is GLUT4 that accounts for the augmentation of transporters after treatment with insulin. In fact, it has been shown in adipocytes that GLUT4 is preferentially translocated in response to insulin and that GLUT1 contributes little to the effect of the hormone in adipocytes (24), as well as in skeletal muscle (25). Thus, it appears reasonable to assume that the differential sensitivity to forskolin of the basal and insulin-stimulated states reflects the different affinities of the GLUT1 and GLUT4 transporters for forskolin.

The present findings have some implications for current methods of assessment of the number of glucose transporters in tissues. According to our data, both GLUT1 and GLUT4 are labeled when cytochalasin B is used in the binding assay. Thus, in all previous studies on adipocyte membranes the sum of these transporters has been determined. If forskolin is used as a ligand, GLUT4 will be labeled preferentially. Therefore, the number of transporters in adipocyte plasma membranes assayed with forskolin is somewhat lower than that assayed with cytochalasin B, with the difference probably representing the amount of GLUT1. Similarly, photolabeling of the GLUT1 requires much higher concentrations of tritiated forskolin than of cytochalasin B (8); tritiated forskolin could thus be used to selectively label the GLUT4 in adipocytes. The forskolin-derived photolabel IAPS-forskolin has been shown to label glucose transporters in both erythrocytes and adipocytes (26) and failed to label glucose transporters in liver membranes.[1] In contrast to forskolin, IAPS-forskolin does not discriminate GLUT1 and GLUT4, as judged from the relative efficiencies of photolabeling in adipocytes and erythrocytes (26).[1] Furthermore, the concentrations of IAPS-forskolin producing half-maximal inhibition of glucose transport in erythrocytes were similar to those of cytochalasin B and >1 order of magnitude higher than those of forskolin (9). Thus, in contrast to forskolin, IAPS-forskolin appears to bind to GLUT1 with high affinity and can be used in order to quantitate the ratio of GLUT4 versus GLUT1 in adipocytes, after a separate immunoprecipitation of both labeled transporters (24).

A cDNA clone encoding a glucose transporter-like protein different from the previously cloned species was isolated from a fetal muscle cDNA library and has been designated GLUT3 (27). Levels of mRNA of this transporter were highest in brain. Low levels were detected in liver and subcutaneous fat; no expression of mRNA was detected in adult skeletal muscle. We are unable to assess the abundance of the protein in our membrane preparations, because we have no specific antiserum for GLUT3. Therefore, it can only be speculated whether GLUT3 binds cytochalasin B and forskolin with high affinity. However, its presence would not change the conclusions on the binding affinities of GLUT1, GLUT2, and GLUT4, which are mainly based on the data from erythrocyte, liver, and adipocyte membranes.

It has to be considered whether the differences in the binding affinities of GLUT1 and GLUT4 reflect the structural variation of a single binding site that decreases the affinity for forskolin but not the affinity for cytochalasin B. Alternatively, the present data might be explained by binding of the ligands to more than one site. Based on previous data (5), we have assumed a competitive type of inhibition of cytochalasin B binding by forskolin and have calculated K_I values for this inhibition as well as for the inhibition of forskolin binding by cytochalasin B. The data, however, are not entirely compatible with the simple model of competitive inhibition at a single site. Most strikingly, in adipocyte and muscle membranes and the K_D values of [^{3}H]cytochalasin B binding were clearly higher than the corresponding K_I values of cytochalasin B in inhibiting [^{3}H]forskolin binding (Table 1). Conversely, the K_I values of forskolin in inhibiting [^{3}H]cytochalasin binding in heart and adipocytes were higher than the corresponding K_D values of [^{3}H]forskolin binding (Table 1). In other words, lower affinities were obtained when tritiated cytochalasin B was used as the tracer instead of tritiated forskolin. This discrepancy might be reconciled with the assumptions 1) that binding of one molecule of ligand takes place at more than one site of the glucose transporter, 2) that a site with high affinity for cytochalasin B but not for forskolin is present in both GLUT1 and GLUT4, and 3) that a site with high affinity for forskolin is present only in GLUT4. If the interaction of the ligands is competitive at some sites and noncompetitive at others, K_D and K_I values would depend on the tracer ligand used in the binding assay. Supporting these assumptions, a model predicting at least four ligand binding sites has previously been suggested for the binding of cytochalasin B to GLUT1, on the basis of the three-dimensional structures of cytochalasins and their structure-activity relationships (28). Furthermore, it has recently been

[1] B. Hellwig and H. G. Joost, unpublished observations.

shown that partial tryptic digestion of GLUT1 reduces its binding affinity for cytochalasin B without affecting the number of sites (29). This finding suggests that it is possible to separate at least two sites of the transporter that participates in the binding of one molecule of cytochalasin B.

Acknowledgments

The authors are indebted to Dr. H. Metzger, Hoechst AG (Frankfurt), for providing the unlabeled forskolin, to Dr. S. W. Cushman, National Institutes of Health (Bethesda, MD), for a gift of the anti-GLUT1 antiserum (α-CT1), to Dr. B. Thorens, Whitehead Institute (Cambridge, MA), for a gift of the anti-GLUT2 antiserum, to Dr. W. E. Schmidt (Göttingen) for the synthesis of a peptide corresponding to the carboxyl terminus of GLUT4, and to Christoph Schmitz-Salue for expert technical assistance.

References

1. Seamon, K. B., and J. W. Daly. Forskolin, cyclic AMP and cellular physiology. *Trends Pharmacol. Sci.* **4**:120–123 (1983).
2. Daly, J. W. Forskolin, adenylate cyclase, and cell physiology: an overview. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**:81–89 (1984).
3. Kashiwagi, A., T. P. Hueckstead, and J. E. Foley. The regulation of glucose transport by cAMP stimulators via three different mechanisms in rat and human adipocytes. *J. Biol. Chem.* **258**:13685–13692 (1983).
4. Sergeant, S., and H. D. Kim. Inhibition of 3-*O*-methylglucose transport in human erythrocytes by forskolin. *J. Biol. Chem.* **662**:245–253 (1985).
5. Joost, H. G., H. J. Steinfelder. Forskolin inhibits insulin-stimulated glucose transport in rat adipose cells by a direct interaction with the glucose transporter. *Mol. Pharmacol.* **31**:279–283 (1987).
6. Joost, H. G., A. D. Habberfield, I. A. Simpson, A. L. Laurenza, and K. B. Seamon. Activation of adenylate cyclase and inhibition of glucose transport in rat adipocytes by forskolin analogues: structural determinants for distinct sites of action. *Mol. Pharmacol.* **33**:449–453 (1988).
7. Kim, H. D., S. Sergeant, and D. S. Shukla. Glucose transport in human platelets and its inhibition by forskolin. *J. Pharmacol. Exp. Ther.* **236**:585–589 (1986).
8. Shanahan, M. F., D. P. Morris, and B. M. Edwards. [^{3}H]Forskolin: direct photoaffinity labeling of the erythrocyte D-glucose transporter. *J. Biol. Chem.* **262**:5978–5984 (1987).
9. Wadzinsky, B. E., M. F. Shanahan, and A. E. Ruoho. Derivatization of the human erythrocyte glucose transporter using a novel forskolin photoaffinity label. *J. Biol. Chem.* **262**:17683–17689 (1987).
10. Pilch, P. F. Glucose transporters: what's in a name? (editorial). *Endocrinology* **126**:3–5 (1990).
11. Steinfelder, H. J., S. Schramm, and H. G. Joost. Prostaglandin E (2) differentiates between two forms of glucose transport inhibition by lipolytic agents. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336**:105–110 (1987).
12. Steinfelder, H. J., and H. G. Joost. Inhibition of glucose transport in rat adipocytes by nucleoside transport inhibitors. *FEBS Lett.* **227**:215–219 (1988).
13. Baldwin, S. A., J. M. Baldwin, and G. E. Lienhard. Monosaccharide transporter of the human erythrocyte: characterization of an improved preparation. *Biochemistry* **21**:3836–3842 (1982).
14. Weber, T. M., H. G. Joost, I. A. Simpson, and S. W. Cushman. Methods for assessment of glucose transport activity and the number of glucose transporters in isolated rat adipose cells and membrane fractions, in *Receptor Biochemistry and Methodology. Insulin Receptors. B. Biological Responses, and Comparison to the IGF-I Receptor* (C. R. Kahn and L. Harrison, eds.). Alan R. Liss, Inc., New York, 171–187 (1988).
15. Joost, H. G., T. M. Weber, and S. W. Cushman. Qualitative and quantitative comparison of glucose transport activity and glucose transporter concentration in plasma membranes from basal and insulin-stimulated rat adipose cells. *Biochem. J.* **249**:155–161 (1988).
16. Haspel, H. C., M. G. Rosenfeld, and O. M. Rosen. Characterization of antisera to a synthetic carboxyl-terminal peptide of the glucose transporter protein. *J. Biol. Chem.* **263**:398–403 (1988).
17. Thorens, B., J. S. Flier, H. F. Lodish, and B. B. Kahn. Expression of two glucose transporters in rat liver is differentially regulated by fasting and refeeding and by diabetes and insulin treatment. *Diabetes* **39**:712–719 (1990).
18. Cheng, Y., and W. H. Prusoff. Relationship between inhibition constant (K_i) and concentration of inhibitor which causes 50 per cent inhibition of an enzymatic reaction (I_{50}). *Biochem. Pharmacol.* **22**:3099–3108 (1973).
19. James, D. E., R. Brown, J. Navarro, and P. F. Pilch. Insulin regulatable tissues express a unique insulin-sensitive glucose transport protein. *Nature (Lond.)* **333**:183–185 (1988).
20. Lavis, V. R., D. L. Lee, and S. Shenolikar. Evidence that forskolin binds to the glucose transporter of human erythrocytes. *J. Biol. Chem.* **262**:14571–14575 (1987).
21. Axelrod, J. D., and P. F. Pilch. Unique cytochalasin B binding characteristics of the hepatic glucose carrier. *Biochemistry* **22**:2222–2227 (1983).
22. Ciaraldi, T. P., R. Horuk, and S. Matthaei. Biochemical and functional characterization of the rat liver glucose-transport system: comparison with the adipocyte glucose-transport system. *Biochem. J.* **240**:115–123 (1986).
23. Klip, A., T. Ramlal, A. G. Donan, P. J. Bilan, and K. L. Skorecki. Inhibition by forskolin of insulin-stimulated glucose transport in L6 muscle cells. *Biochem. J.* **255**:1023–1029 (1988).
24. Zorzano, A., W. Wilkinson, N. Kotliar, G. Thoidis, B. E. Wadzinsky, A. E. Ruoho, and P. E. Pilch. Insulin-regulated glucose uptake in rat adipocytes is mediated by two transporter isoforms present in at least two vesicle populations. *J. Biol. Chem.* **264**:12358–12363 (1989).
25. Douen, A. G., T. Ramlal, S. Rastogi, P. J. Bilan, G. D. Cartee, M. Vranic, J. O. Holloszy, and A. Klip. Exercise induces recruitment of the 'insulin-responsive glucose transporter': evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. *J. Biol. Chem.* **265**:13427–13430 (1990).
26. Wadzinsky, B. E., M. F. Shanahan, R. B. Clark, and A. E. Ruoho. Identification of the glucose transporter in mammalian cell membranes with a ^{125}I-forskolin photoaffinity label. *Biochem. J.* **255**:983–990 (1988).
27. Kayano, T., H. Fukumoto, R. L. Eddy, Y. S. Fan, M. G. Byers, T. B. Shows, and G. I. Bell. Evidence for a family of human glucose transporter-like proteins: sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues. *J. Biol. Chem.* **263**:15245–15248 (1988).
28. Griffin, J. E., A. L. Rampal, and C. Y. Jung. Inhibition of glucose transport in human erythrocytes by cytochalasins: a model based on diffraction studies. *Proc. Natl. Acad. Sci. USA* **79**:3759–3763 (1982).
29. Cairns, M. T., J. Alvarez, M. Panico, A. F. Gibbs, H. R. Morris, D. Chapman, and S. A. Baldwin. Investigation of the structure and function of the human glucose transporter by proteolytic digestion. *Biochim. Biophys. Acta* **905**:295–310 (1987).

Send reprint requests to: Dr. Hans G. Joost, Institut für Pharmakologie und Toxikologie der RWTH Aachen, Wendlingweg 2, D-5100 Aachen, Federal Republic of Germany.