Evidence That Forskolin Binds to the Glucose Transporter of Human Erythrocytes*

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Binding of [4-3H]cytochalasin B and [12-3H]forskolin to human erythrocyte membranes was measured by a centrifugation method. Glucose-displaceable binding of cytochalasin B was saturable, with $K_D = 0.11 \mu M$, and maximum binding $\approx 550$ pmol/mg of protein. Forskolin inhibited the glucose-displaceable binding of cytochalasin B in an apparently competitive manner, with $K_I = 3 \mu M$. Glucose-displaceable binding of [12-3H]forskolin was also saturable, with $K_D = 2.6 \mu M$ and maximum binding $\approx 400$ pmol/mg of protein. The following compounds inhibited binding of [12-3H]forskolin and [4-3H]cytochalasin B equivalently, with relative potencies parallel to their reported affinities for the glucose transport system: cytochalasins A and D, dihydrocytochalasin B, L-rhamnose, L-glucose, D-galactose, D-mannose, D-glucose, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, phloretin, and phlorizin. A water-soluble derivative of forskolin, 7-hemisuccinyl-7-desacetylforskolin, displaced equivalent amounts of [4-3H]cytochalasin B or [12-3H]forskolin. Rabbit erythrocyte membranes, which are deficient in glucose transporter, did not bind either [4-3H]cytochalasin B or [12-3H]forskolin in a glucose-displaceable manner. These results indicate that forskolin, in concentrations routinely employed for stimulation of adenylyl cyclase, binds to the glucose transporter. Endogenous ligands with similar specificities could be important modulators of cellular metabolism.

It has recently been shown that forskolin, a diterpene activator of adenylyl cyclase (1), strongly inhibits facilitated diffusion of glucose in human erythrocytes (2) and blood platelets (3) and in rat or human adipocytes (4, 5). Published data (2–5) have suggested that this effect of forskolin is not entirely mediated by cyclic AMP. Herein we present evidence that forskolin interacts directly with the hexose transporter of human erythrocyte membranes. These results have important implications for the coordinate regulation of metabolism in hormonally responsive tissues.

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EXPERIMENTAL PROCEDURES

Materials—[4-3H]Cytochalasin B, [12-3H]forskolin, and [U-14C]sucrose were purchased from Du Pont-New England Nuclear. The [12-3H]forskolin was repurified by preparative thin-layer chromatography on silica gel, using two solvent systems: (a) cyclohexane/ethyl acetate (3:1, v/v) and (b) chloroform/methanol (98:2, v/v).

Forskolin and 7-O-hemisuccinyl-7-desacetylforskolin were obtained from Behring Diagnostics. Cytochalasin B, dihydrocytochalasin B, cytochalasin A, cytochalasin D, cytochalasin E, and 3-O-methyl-D-glucose were from Aldrich. D-Sorbitol, L-glucose, L-rhamnose, D-galactose, 2-deoxy-D-glucose, D-mannose, phlorizin, and phloretin were from Sigma. D-Glucose was from J. T. Baker Chemical Co. Sodium dodecyl sulfate was from Bio-Rad.

Preparation of Erythrocyte Membranes—Human erythrocytes were obtained from freshly drawn blood of normal adult donors. Essentially hemoglobin-free membranes ("ghosts") were prepared by the method of Dodge et al. (6). The membranes were suspended in 5 mM sodium phosphate buffer, pH 8.0, at concentrations of 1.6–2.4 mg of protein/ml and stored in liquid nitrogen.

Membrane Protein Determination—Membranes were solubilized in 0.5 M NaOH at 50 °C overnight and then assayed by the method of Lowry et al. (7) with crystalline bovine serum albumin as standard.

Binding Assays—Binding of [4-3H]cytochalasin B or [12-3H]forskolin to erythrocyte membranes was determined by a centrifugation method. Incubation mixtures contained tritiated ligand (0.03 μCi of [4-3H]cytochalasin B or 0.35 μCi of [12-3H]forskolin), nonradioactive competing ligand as appropriate, monosaccharide (500 mM D-glucose or 500 mM sorbitol), and erythrocyte membranes (0.15–0.2 mg of protein/ml) in 5 mM sodium phosphate buffer, pH 8.0, at a final volume of 1.0 ml. In some experiments, other monosaccharides replaced p-glucose. Stock solutions of forskolin and cytochalasin B were dissolved in methanol and ethanol, respectively. Appropriate vehicle controls were included in each experiment, so that the final concentration of alcohol in all tubes was 2.1% (v/v). All tubes contained 19 μCi cytochalasin E, which has been shown to compete with [4-3H]cytochalasin B for binding to nontransporter-related sites, without affecting its binding to the glucose transporter (8–10). In preliminary experiments, we confirmed that cytochalasin E reduced nonspecific (glucose-nondisplaceable) binding of [12-3H]forskolin to the membranes, without affecting glucose-displaceable binding. Therefore, we routinely employed cytochalasin E in the incubations. All incubations were performed in triplicate.

Incubations were started by addition of 100 μl of membrane suspension to tubes containing all the other components, with mixing. In preliminary experiments we determined that binding of either ligand was stable with incubation times from 5 to 15 min at room temperature. The standard procedure was to incubate for 5 min at room temperature, followed by centrifugation for 3 min in a microcentrifuge. After centrifugation, 500 μl of supernatant was transferred to a scintillation vial, and the rest of the supernatant was carefully aspirated off the membrane pellet. Each pellet was then dissolved in 50 μl of sodium dodecyl sulfate (11 mg/ml) and transferred to another scintillation vial. The incubation tube was then rinsed with 50 μl of water and the rinse added to the vial containing the dissolved membrane. The vials were counted in a liquid scintillation counter, with Hydrofluor (National Diagnostics, Inc.) as the scintillation fluid.

Concentrations of bound and free ligands were determined from the counts in the vials containing membranes and supernatants, respectively. The counting efficiency of 3H in the vials containing...
supernatants was from 88 to 90% that in the vials with membranes. Also, from 3 to 9% free ligand was trapped in the pellets, as determined by parallel incubations with \(^{14}C\) glucose, which does not enter human erythrocytes (11). All the data have been appropriately corrected for counting efficiency and for trapping, which were measured independently in each day's experiment. Glucose-displaceable binding of ligand was calculated as the difference between the amount bound in the presence of 500 mM sorbitol (total binding) and that bound in the presence of 500 mM D-glucose. Binding in the presence of 500 mM D-glucose, which amounted to 6% total counts for \([4-^3H]\) cytochalasin B and 4% counts for \([12-^3H]\) forskolin, was nonsaturable and considered not to be specific for the glucose transporter. The relative amount of nonspecific binding increased with concentration of ligand, varying from 10 to 50% total binding over the range of 20 nM to 2 \(\mu M\) \([4-^3H]\) cytochalasin B, and from 13 to 50% total binding over the range of 1 nM to 30 \(\mu M\) \([12-^3H]\) forskolin.

The data were analyzed by the "LIGAND" program of Munson and Rodbard (12), kindly provided by Drs. Peter Munson and Michael Beveridge of the National Institutes of Health.

RESULTS

Glucose-displaceable binding of cytochalasin B to erythrocyte membranes was saturable. The Scatchard plot (13), shown in Fig. 1, indicates a single class of binding sites, with \(B_{max} \approx 500\, \text{pmol/mg of protein}\) and \(K_D = 0.11\, \mu M\). These results are consistent with published values (14–16).

The Scatchard plot of glucose-displaceable binding of \([12-^3H]\) forskolin to erythrocyte membranes is shown in Fig. 2. The data suggest the existence of nonsaturable binding even after correction for displacement by glucose. After correction for residual nonsaturable binding (12, 17), we obtained the following coefficients: \(K_D = 2.6\, \mu M\), maximal binding \(\approx 400\, \text{pmol/mg of protein}\).

As a check on the accuracy of the above methods for determination of nonspecific binding, we also analyzed the raw data without correction for displacement of \(^3H\) ligand by D-glucose. In this analysis, nonspecific binding was treated as a statistic to be estimated by the curve-fitting algorithms of the "LIGAND" program. This method yielded results essentially identical with those derived from the corrected data: for \([4-^3H]\) cytochalasin B, nonspecific binding = 5.2% total counts, \(K_D = 0.15\, \mu M\), \(B_{max} = 650\, \text{pmol/mg of protein}\); for \([12-^3H]\) forskolin, nonspecific binding = 4.1% total counts, \(K_D = 2.3\, \mu M\), \(B_{max} = 590\, \text{pmol/mg of protein}\).

Glucose-displaceable binding of \([4-^3H]\) cytochalasin B was inhibited by forskolin in an apparently competitive manner, with \(K_I = 3\, \mu M\) (Fig. 3). However, binding of \([4-^3H]\) cytochalasin B in the presence of 500 mM D-glucose, presumably reflecting binding to nontransporter-related sites (8), was only minimally (less than 12%) displaced by 50 \(\mu M\) forskolin (data not shown).

Cytotochalasin B displaced forskolin from the membranes. Glucose-displaceable binding of between 0.4 and 3 \(\mu M\) \([12-^3H]\) forskolin was half-maximally inhibited by 0.2–0.6 \(\mu M\) cytochalasin B (data not shown), concentrations which approximate the \(K_D\) for binding of cytochalasin B. As shown in Table I, other cytochalasins displaced \([12-^3H]\) forskolin and \([4-^3H]\) cytochalasin B equivalently, with an order of potency parallel to their published relative potencies as inhibitors of glucose transport (8).

We also investigated displacement of forskolin and cytochalasin B by a water-soluble derivative of forskolin, 7-O-hemisuccinyl-7-desacetylforskolin. The results, in Table II,
**TABLE I**

Displacement of cytochalasin B and forskolin by various cytochalasins

Erythrocyte membranes were incubated for 5 min with 0.2 μM [4-3H]cytochalasin B or 3 μM [12-3H]forskolin and nonradioactive cytochalasins as shown in the table. Binding was determined as described under "Experimental Procedures." Results are expressed as pmol bound/mg of protein: mean of triplicate determinations for a representative experiment.

| Competing cytochalasin | Glucose-displaceable cytochalasin B | Glucose-displaceable forskolin |
|------------------------|-------------------------------------|-------------------------------|
| None                   | 236 0                               | 296 0                         |
| Cytochalasin B         |                                     |                               |
| 1 μM                   | 58 -76 99 -66                      |                               |
| 10 μM                  | 2 99 11 -96                       |                               |
| Cytochalasin A         |                                     |                               |
| 1 μM                   | 116 -51 148 -50                    |                               |
| 10 μM                  | 10 -96 0 -100                     |                               |
| Dihydrocytochalasin B  |                                     |                               |
| 1 μM                   | 201 -15 282 -5                     |                               |
| 10 μM                  | 200 -11 218 -26                    |                               |
| Cytochalasin D         |                                     |                               |
| 1 μM                   | 219 -7 309 4                       |                               |
| 10 μM                  | 227 -4 296 -3                      |                               |

**TABLE II**

Displacement of [4-3H]cytochalasin B or [12-3H]forskolin by 7-hemisuccinyl-7-desacetylforskolin

Erythrocyte membranes were incubated for 5 min with 0.2 μM [4-3H]cytochalasin B or 1.9 μM [12-3H]forskolin and various concentrations of 7-hemisuccinyl-7-desacetylforskolin, as shown in the table. Binding was determined as described under "Experimental Procedures." Results are expressed as pmol bound/mg of protein: mean of triplicate determinations for a representative experiment.

| [7-Hemisuccinyl-7-desacetylforskolin] | Glucose-displaceable cytochalasin B | Glucose-displaceable forskolin |
|-------------------------------------|-------------------------------------|-------------------------------|
| μM                                  | pmol/mg protein | % change | pmol/mg protein | % change |
| None                                | 237 0           | 193 0    |                  |          |
| 1.92                                | 236 -1          | 207 7    |                  |          |
| 4.8                                 | 208 -12         | 124 -36  |                  |          |
| 12                                  | 196 -17         | 119 -39  |                  |          |
| 30                                  | 142 -40         | 151 -22  |                  |          |
| 75                                  | 74 -69          | 54 -72   |                  |          |

showed that the analog displaced equivalent amounts of [12-3H]forskolin or [4-3H]cytochalasin B, over a wide range of concentrations.

Binding of cytochalasin B to hexose transporter is inhibited by sugars in approximate proportion to their apparent affinities for transport (15, 18). We compared a series of hexoses for their abilities to displace [4-3H]cytochalasin B or [12-3H]forskolin. As shown in Table III, the relative orders of potency and degrees of inhibition were the same for either labeled ligand: L-rhamnose and L-glucose were ineffective; D-galactose and D-mannose were of intermediate potency; and D-glucose, 2-deoxy-D-glucose, and 3-O-methyl-D-glucose were the most powerful inhibitors of binding. This order of potency closely parallels the reported affinities of these sugars for the hexose transport system (19).

Phloretin inhibits hexose transport in human erythrocytes (19, 20) and has previously been shown to inhibit binding of cytochalasin B to partially purified hexose transporter (8, 16). As shown in Table III, we found that phloretin displaced equivalent amounts of [4-3H]cytochalasin B or [12-3H]forskolin from erythrocyte membranes. Phlorizin, a glucosidic ana-

**LOG OF PHLORETIN WITH LITTLE OR NO TRANSPORT-INHIBITORY ACTIVITY IN ERYTHROCYTES (19, 21), DID NOT DISPLACE EITHER LIGAND.**

In an attempt to elucidate the binding specificities of [12-3H]forskolin, we studied membranes from rabbit erythrocytes, which transport glucose slowly and in a nonspecific manner (19, 22, 23) and lack transport-related binding sites for cytochalasin B (22, 24), presumably because they are deficient in hexose transporter. Our results, in Fig. 4, A and B, show that binding of both cytochalasin B and forskolin by these membranes was linear and apparently nonsaturable, with no displacement by glucose. The total binding of either ligand to rabbit membranes was quantitatively the same as the nonspecific binding to human membranes.

**DISCUSSION**

Stereospecific facilitated diffusion of glucose into mammalian cells is inhibited by the fungal metabolite cytochalasin B (8, 25), an effect which seems to be mediated by binding to the hexose transporter (8, 14–16, 25). Recently published work has indicated that forskolin, thought previously to be a specific activator of adenylate cyclase, inhibits glucose transport and competes with cytochalasin B for binding to membranes from human erythrocytes (2, 26) and rat adipocytes (5). Our data elucidate further the binding interactions between forskolin and cytochalasin B.

In this work, we have minimized binding of radiolabeled log of phloretin with little or no transport-inhibitory activity in erythrocytes (19, 21), did not displace either ligand.

**Inhibition of binding of cytochalasin B and forskolin by sugar analogs**

Erythrocyte membranes were incubated for 5 min with 0.2 μM [4-3H]cytochalasin B or 3 μM [12-3H]forskolin and nonradioactive analogs as shown in the table. Binding was determined as described under "Experimental Procedures." Results are expressed as percentage of binding in the presence of 500 mM sorbitol: mean ± S.E. for three separate experiments (triplicate incubations in each experiment).

| Competing sugar     | Relative affinity* | Cytochalasin B | Forskolin |
|---------------------|--------------------|----------------|-----------|
| Sorbitol            |                    | 100            | 100       |
| L-Glucose 5 mM      | >3 M               | 102 ± 2        | 98 ± 16   |
| 50 mM               |                    | 103 ± 2        | 87 ± 8    |
| L-Rhamnose 5 mM     | ≈3 M               | 100 ± 2        | 90 ± 5    |
| 50 mM               |                    | 100 ± 2        | 98 ± 10   |
| D-Galactose 5 mM    |                    | 98 ± 1         | 95 ± 13   |
| 50 mM               |                    | 90 ± 1         | 74 ± 12   |
| D-Mannose 5 mM      |                    | 96 ± 1         | 87 ± 18   |
| 50 mM               |                    | 77 ± 2         | 75 ± 2    |
| D-Glucose 5 mM      |                    | 94 ± 1         | 86 ± 1    |
| 50 mM               |                    | 52 ± 2         | 75 ± 12   |
| 500 mM              |                    | 14 ± 1         | 19 ± 7    |
| D-3-O-Methylglucose |                    | 89 ± 1         | 84 ± 12   |
| 5 mM                |                    | 52 ± 3         | 57 ± 13   |
| 50 mM               |                    | 85 ± 1         | 90 ± 19   |
| 2-Deoxyglucose 4.5 mM |                | 41 ± 1         | 46 ± 6    |
| Phloretin 5 μM      | 72 ± 4             | 60 ± 4         |           |
| 50 μM               |                    | 28 ± 4         | 26 ± 14   |
| Phlorizin 5 μM      | 96 ± 2             | 105 ± 21       |           |
| 50 μM               |                    | 89 ± 4         | 81 ± 22   |

* Relative affinities as transport substrates or inhibitors according to the review of LeFevre (19).
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**FIG. 4.** Binding of [4-3H]cytochalasin B and [12-3H]forskolin to human and rabbit erythrocyte membranes. Incubations and determination of binding were as described under "Experimental Procedures." Each point represents the mean of triplicate incubations and one with rabbit membranes. Glucose (total binding); 0, nonspecific binding to rabbit membranes. Binding to human membranes in the absence of glucose (total binding); +, binding to human membranes in the presence of 500 mM D-glucose (nonspecific binding); ○, total binding to rabbit membranes; Δ, nonspecific binding to rabbit membranes. A, [4-3H]cytochalasin B. Data are shown from 10 experiments with human membranes and one with rabbit membranes. B, [12-3H]forskolin. Data are shown from 6 experiments with human membranes and one with rabbit membranes.

ligands to sites not associated with glucose transport (8, 9, 24) by correction for extraneous binding in the presence of 500 mM D-glucose, and by inclusion of nonradioactive cytochalasin E, which competes with cytochalasin B for nontransport-related sites (8–10), in all the incubations. Under these conditions, forskolin exhibited a number of characteristics suggestive of direct interaction with hexose transporter.

First, binding of [12-3H]forskolin was apparently saturable, with a dissociation constant comparable to the $K_D$ for displacement of [4-3H]cytochalasin B. Second, cytochalasins A and D, dihydrocytochalasin B, phloretin, and phlorizin all displaced [12-3H]forskolin with relative potencies parallel to those observed for displacement of [4-3H]cytochalasin B and previously reported for inhibition of hexose transport (8, 19, 21, 24). Third, various hexoses displaced [12-3H]forskolin with potencies proportional to their apparent affinities as substrates for transport (19). Fourth, inverse plots of [4-3H]cytochalasin B binding suggested a competitive mode of inhibition for forskolin. Finally, no glucose-displaceable binding of either [4-3H]cytochalasin B or [12-3H]forskolin was observed in adult rabbit erythrocyte membranes, which effectively lack stereospecific hexose transporter (19, 22–24).

The Scatchard plots (Figs. 1 and 2) suggest that the number of glucose transporter-related [12-3H]forskolin-binding sites ($409 \text{ pmol/mg of protein}$) is approximately equal to that for cytochalasin B ($550 \text{ pmol/mg of protein}$). Because the limit of solubility of forskolin, approximately $30 \mu\text{M}$, is less than 5-fold greater than the $K_D$ for binding, it was necessary to extrapolate from data obtained at less than saturating concentrations, a procedure which leads to inaccuracy (27). However, the nonextrapolated data also suggest equal numbers of sites for forskolin and cytochalasin B. For example, the number of glucose-displaceable forskolin-binding sites at the $K_D$ for competition with cytochalasin B was $260 \text{ pmol/mg of protein}$, compared with half-maximal binding of 275 pmol/mg of protein for cytochalasin B. Also, the nonradioactive cytochalasins and 7-hemisuccinyl-7-desacetylforskolin displaced equivalent amounts of [4-3H]cytochalasin B or [12-3H]forskolin for data pooled from all experiments with nonradioactive analogs, the regression of pmol of [12-3H]forskolin displaced on pmol of [4-3H]cytochalasin B displaced had a slope of 1.05 ($r = 0.897$). We infer that there is a 1:1 ratio of forskolin/cytochalasin B binding sites. One would not have anticipated this binding ratio, if forskolin were displacing cytochalasin B by a cascade mechanism involving cyclic AMP-dependent protein phosphorylation. Indeed, from currently available data, it seems that none of the components of the adenylate cyclase system is sufficiently abundant in erythrocytic membranes to have contributed measurably to the observed level of [12-3H]forskolin binding.

The $K_D$ for displacement of cytochalasin B by forskolin (3 $\mu\text{M}$) lies within the range of reported half-maximal concentrations (2–7.5 $\mu\text{M}$) for inhibition of hexose transport in human platelets (3) and erythrocytes (2) and for inhibition of glucose transport and oxidation in rat adipocytes (5, 31). Also, the $K_D$ for binding of [12-3H]forskolin approximates that reported (1–2 $\mu\text{M}$) for binding of forskolin to low affinity sites on rat brain and liver membranes (32, 33). In contrast, Joost and Steinfelder (5) recently reported a nearly 50-fold greater potency of forskolin for displacement of cytochalasin B from adipocyte membrane vesicles ($K_D = 200 \text{ nM}$), with an equivalent half-maximal dose for inhibition of 3-O-methylglucose transport. The reasons for the discrepancy with our erythrocyte data are yet unknown. Further work with isolated components will be required in order to determine whether forskolin and cytochalasin B compete for the same binding site, and to clarify the relationships of the high and low affinity binding sites for forskolin (reviewed in Ref. 34) to the hexose transporter and to adenylate cyclase, in various tissues.

Because human erythrocytic plasma membranes are relatively rich in glucose transporter (24, 35, 36) and poor in adenylate cyclase catalytic activity, they provide a useful system for the study of actions of forskolin unrelated to activation of adenylate cyclase. Photolabeling with cytochalasin B (25, 37, 38), immunologic methods (39–40), and, most

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1 The highest catalytic activity so far reported for unfractionated human erythrocytic membranes has been 6 pmol/min/mg of protein (28); assuming this is only 0.1% of maximal activity, with a turnover number of 1000 min$^{-1}$ (29), this would represent 6 pmol of catalytic units/mg of protein. Similarly, quantitative ADP-ribosylation has yielded estimated levels of 1 and 4 pmol/mg of protein for the Ns and Nt (stimulatory and inhibitory guanyl nucleotide-binding) regulatory subunits, respectively (30). These numbers are 2 orders of magnitude lower than the level of binding we observed.
recently, mRNA hybridization studies (41) all have demonstrated similarities between the glucose transporter of human erythrocytes and those of human placenta (25), fibroblasts (39), and adipocytes (35), rat cerebral microvessels (37), brain (41), skeletal muscle (38), basolateral renal cortical membranes (42, 43), and adipocytes (36, 40, 41), murine reticulocytes (40) and preadipocytes (39), and Friend cells (40). Therefore, there is good reason to think that forskolin will also bind to transporter from other tissues.

Forskolin has hitherto been thought to act specifically on adenylate cyclase (reviewed in Ref. 1). The finding that it also binds to transporter from other tissues. In order to clarify the roles of cyclic AMP and glucose transport on control of metabolism, it will be necessary to employ agents that selectively interact with one or the other of these two systems. Furthermore, we speculate that there may exist cellular analogs of forskolin which could reciprocally modulate cyclic AMP generation and glucose transport. In this regard, we note with great interest the recent reports that mediators of insulin action can be derived from lipid-soluble precursors in plasma membranes (42, 43) and that insulin (44, 45) and catecholamines (46) can rapidly alter the activity of glucose transporters in adipocyte plasma membranes. Intramembranal lipids would be attractive candidates for the putative endogenous ligands to cytochalasin B/forskolin binding site(s) on the hexose transporter.

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