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CHARACTERIZATION OF CYTOCHALASIN B BINDING TO ADULT RAT LIVER PARENCHYMAL CELLS IN PRIMARY CULTURE

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Summary

The characterization of cytochalasin B binding and the resulting effect on hexose transport in rat liver parenchymal cells in primary culture were studied. The cells were isolated from adult rats by perfusing the liver in situ with collagenase and separating the hepatocytes from the other cell types by differential centrifugation. The cells were established in primary culture on collagen-coated dishes. The binding of [4-^{3}H]cytochalasin B and transport of 3-*O*-methyl-D-[^{14}C]glucose into cells were investigated in monolayer culture followed by digestion of cells and scintillation counting of radioactivity. The binding of cytochalasin B to cells was rapid and reversible with association and dissociation being essentially complete within 2 min. Analysis of the kinetics of cytochalasin B binding by Scatchard plots revealed that binding was biphasic, with the parenchymal cell being extremely rich in high-affinity binding sites. The high-affinity site, thought to be the glucose-transport carrier, exhibited a K_D of $2.86 \cdot 10^{-7}$ M, while the low-affinity site had a K_D of $1.13 \cdot 10^{-5}$ M. Sugar transport was monitored by 3-*O*-methyl-D-glucose uptake and it was found that cytochalasin B (10^{-5} M) drastically inhibited transport. However, D-glucose (10^{-5} M) did not displace cytochalasin B, and cytochalasin E, which does not inhibit transport, was competitive for cytochalasin B at only the low-affinity site, demonstrating that the cytochalasin B inhibition of sugar transport occurs at the high-affinity site but that the inhibition is non-competitive in nature. Therefore, the liver parenchymal cells may represent an unusually rich source of glucose-transport system which may be useful in the isolation of this important membrane carrier.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

Introduction

Cytochalasin B [1] is a potent inhibitor of hexose transport [9,10] in many types of eucaryotic cell. In addition, the cytochalasins have drastic effects on cellular morphology and inhibit cell locomotion and division [11–14]. Cytochalasin B-induced changes in cell morphology are independent of the inhibition of transport [4]. Cytochalasin E does not inhibit transport but causes changes in cell morphology at very low drug concentrations. Cytochalasin B effects on transport and morphology are most likely mediated through discrete, separate receptors. Studies on the binding of cytochalasin B to intact cells or plasma membrane have shown that at least two types of receptor with differing affinities are involved [9,19,28]. The highest affinity receptors appear to be part of the hexose-transport system while the lower affinity receptors, which have been found to be more abundant than the higher receptors, are involved with cell morphology or nonspecific binding. Thus, attempts to probe the glucose-transport system by using radioactively labeled cytochalasin B binding to the high-affinity receptor are always compromised by the great excess of low-affinity binding sites.

Our decision to examine the binding of cytochalasin B to intact hepatocytes in primary culture was stimulated by the observation that the morphology of these cells is not altered by concentrations of cytochalasin B that drastically alter morphology of other cell types in culture. In addition, it is well established that glucose transport in liver parenchymal cells is in great excess of what is needed for intracellular metabolism. Indeed, liver cells were thought to be freely permeable to glucose until 1967, when Park et al. [17] were able to demonstrate stereospecific uptake of glucose into liver. Thus, we felt that the adult rat liver parenchymal cells in primary culture might represent an unusually rich source of the glucose-transport system that could be probed by cytochalasin B without interference from the lower affinity sites. In this report, we describe the basic characteristics of cytochalasin B binding to intact hepatocytes.

Materials and Methods

Reagents. [4-^{3}H]Cytochalasin B (11.5 Ci/mmol) and 3-*O*-methyl-D-[^{14}C]-glucose (0.05 mCi/mmol) were purchased from New England Nuclear. Unlabeled cytochalasin B, 3-*O*-methyl-D-glucose, and cytochalasin E were obtained from Sigma Chemical Co.; D-glucose was purchased from Fisher Scientific Co.

Cells and cell culturing. Rat liver parenchymal cells were isolated from adult rat liver essentially by the collagenase-perfusion technique of Bonney et al. [21]. The cells were maintained as a monolayer in Waymouth medium on collagen-coated 35- or 60-mm plates [22,23]. The medium was changed after the cells had been in culture for 3—4 h, by which time approx. 50% of the cells had attached to the culture dish [23]. Additional incubation prior to medium change resulted in no increase in attached cells [23]. Cells used in the studies reported here were in culture for 24 h at the time of experimentation.

[^{3}H]Cytochalasin B binding to intact rat liver parenchymal cells. Assay of [^{3}H]cytochalasin B binding to intact cells was performed by the following procedure. The cells were rinsed with 10 ml of Hanks'/Hepes (26°C) buffer. Following aspiration of the Hanks'/Hepes buffer, 0.8 ml of Hanks'/Hepes buffer containing a known concentration of [^{3}H]cytochalasin B, and any competing small molecules, if specified, was added and the incubation was carried out at 26°C for the designated period of time. At that time, the incubation was terminated by aspirating off the mixture and rinsing the attached cells with 10—15 ml of cold phosphate-buffered (pH 7.4) isotonic saline at 4°C. Continued washing with isotonic saline at 4°C resulted in the loss of about 5% of the remaining counts per 10 ml of wash. Therefore, our values for bound [^{3}H]cytochalasin B may be 5—7.5% low. The cells were then digested in 0.6 ml of 0.1 M NaOH for 15 min. Aliquots were removed for protein determination [24] and scintillation counting.

3-O-Methyl-D-glucose transport in intact cells. The uptake of 3-*O*-methyl-D-glucose by adult rat liver parenchymal cells was performed according to previously published methods [18]. Specifically, cells which were attached to the culture dish, after being washed with Hanks'/Hepes glucose-free medium containing a known concentration of 3-*O*-methyl-D-[^{14}C]glucose at room temperature. At specified times, the medium was removed, the cells were washed in medium (4°C) containing 1 mM phloretin, and digested. To study the effect of cytochalasin B on influx, cytochalasin B at constant concentration was added to the 3-*O*-methyl-D-[^{14}C]glucose-containing mixture.

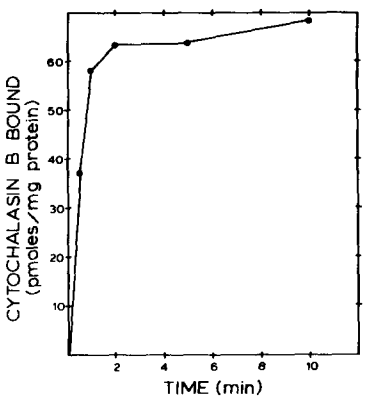
Efflux of 3-*O*-methyl-D-glucose from intact rat liver cells was performed similarly with the modifications that the cells were preloaded to equilibrium with sugar for 30 min and when that mixture was removed, it was replaced with either Hanks'/Hepes buffer alone, or Hanks'/Hepes buffer plus a specific concentration of cytochalasin B. At specified times, this efflux buffer was removed, and the cells quickly washed and treated as previously described.

Other methods. Protein was assayed by using the method of Lowry et al. [24]. Radioactivity was determined by a liquid scintillation spectrometer (LS-9000, Beckman Instruments) using ACS (Amersham/Searle) as the scintillant.

Results

Cytochalasin B binding to intact rat liver hepatocytes. [^{3}H]Cytochalasin B binds rapidly to intact rat liver hepatocytes at 25°C with equilibrium essentially reached within 2 min of incubation (Fig. 1). That this is a reversible association is demonstrated in Fig. 2, which shows that cytochalasin B dissociated from the hepatocyte in a time frame essentially identical with its association, i.e., that within 2 min the dissociation is nearly complete. A large percentage (80%) of this bound radioactivity could be displaced by 10^{-5} M cytochalasin B, implying that greater than 50% of the bound cytochalasin B was associated with the cell in a saturable manner (unpublished results).

In an effort to characterize further the binding of cytochalasin B to intact cells, a Scatchard plot analysis [25] of the binding was carried out (Fig. 3). The results of this experiment indicate that the ratios of bound-to-free [^{3}H]-



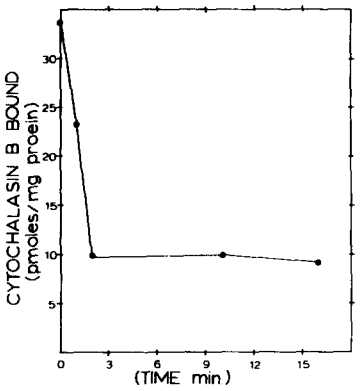


Fig. 1. Rate of association of [^{3}H]cytochalasin B with intact rat liver hepatocytes. The binding of [^{3}H]-cytochalasin B to hepatocytes was determined as indicated in Materials and Methods. The incubations were carried out for the indicated times with 10^{-6} M cytochalasin B. All measurements were performed on duplicate plates.

Fig. 2. Rate of dissociation of [^{3}H]cytochalasin B from intact rat liver hepatocytes. Attached cells were prepared as described in the legend of Fig. 1 and were incubated for 5 min in 0.8 ml of Hanks'/Hepes buffer containing $5 \cdot 10^{-7}$ M cold cytochalasin B and [^{3}H]cytochalasin B at 1 μCi/ml. At the end of this time, the incubation mixture was aspirated off and quickly replaced with 2.0 ml of Hanks'/Hepes buffer. At the specified times, this medium was aspirated and the cells were washed, digested and counted as previously described. The zero-time plate was used as soon as the hot incubation mix was removed and before any Hanks'/Hepes buffer was added. All determinations were on duplicate plates.

cytochalasin B represent essentially a biphasic curve function of the bound cytochalasin B concentration. The biphasic nature of the curve suggests that in the intact rat liver hepatocyte, there exist two distinct classes of binding site for cytochalasin B. One of these, the so-called 'high-affinity' site, exhibited a dissociation constant (K_D) of $2.86 \cdot 10^{-7}$ M. The mean maximum binding capacity of the high-affinity site was calculated to be 245 pmol/mg protein. The values for the 'low-affinity' site were calculated and this binding site exhibits a dissociation constant (K_D) of $1.13 \cdot 10^{-5}$ M, and a mean maximum binding capacity of approx. 1600 pmol/mg protein.

The effect of D-glucose on cytochalasin B binding. Previous work has shown

TABLE I

THE EFFECT OF D-GLUCOSE ON CYTOCHALASIN B BINDING

Cells were prepared as described in the legend of Fig. 1. The cells were then incubated for 5 min in 0.8 ml of Hanks'/Hepes buffer containing the designated constant concentration of cytochalasin B, and varying concentrations of D-glucose. [^{3}H]Cytochalasin B was kept constant in all experiments at 1 μCi/ml. The cells were then washed, digested, and their radioactive content determined. All values are averages of duplicate determinations. Results are expressed as pmol cytochalasin B/mg protein.

Cytochalasin B (M)	Glucose (mM)				
	0	1	10	100	500
$5 \cdot 10^{-7}$	42.14	38.44	39.72	45.35	41.67
$5 \cdot 10^{-8}$	1.22	1.32	1.50	1.31	1.40
$5 \cdot 10^{-9}$	0.126	0.120	0.137	0.330	0.164

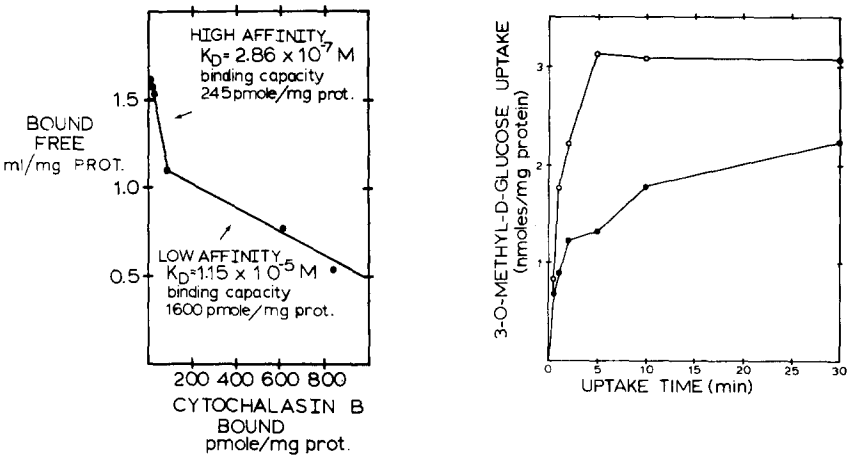


Fig. 3. Scatchard plot analysis of [^{3}H]cytochalasin B binding to intact rat hepatocytes. The binding of [^{3}H]cytochalasin B was determined as in Materials and Methods, and the free cytochalasin B determined after termination of incubation. All determinations were performed in duplicate.

Fig. 4. The effect of cytochalasin B on the time course of the influx of 3-*O*-methyl-D-glucose into intact rat liver cells. The uptake of 1 mM 3-*O*-methyl-D-[^{14}C]glucose was determined in the presence (○) or absence (●) of 10^{-5} M cytochalasin B as described in Materials and Methods. Each point is the average of duplicate plates.

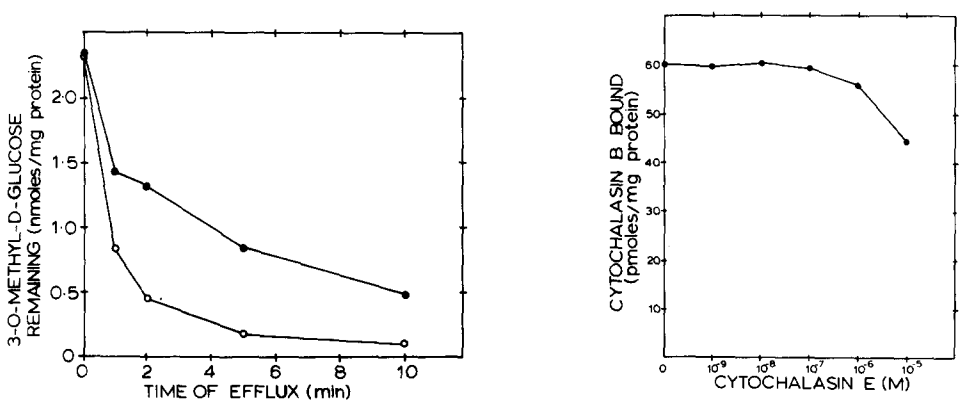


Fig. 5. The effect of cytochalasin B on the time course of the efflux of 3-*O*-methyl-D-glucose from rat liver hepatocytes. The cells were incubated in 0.8 ml of Hanks'/Hepes buffer with 1 mM 3-*O*-methyl-D-glucose, 0.5 μCi/ml 3-*O*-methyl-D-[^{14}C]glucose for 30 min. Following the aspiration of the incubation mix, the cells were quickly washed with 5 ml of cold (4° C) phosphate-buffered isotonic saline. The cells were then bathed in 1.0 ml of Hanks'/Hepes buffer with (●) or without (○) 10^{-5} M cytochalasin B for the appropriate times. The zero-time point was obtained before the 1.0 ml of Hanks'/Hepes was added. Each point represents the average of duplicate plates.

Fig. 6. The effect on cytochalasin E on cytochalasin B binding to intact rat liver hepatocytes. The method involved preparing the cells as described in the legend of Fig. 1, and then incubating the cells for 5 min in 0.8 ml of incubation mix containing a constant concentration of cytochalasin B at 5 · 10^{-7} M, [^{3}H]cytochalasin B at 1 μCi/ml, and varying concentrations of cytochalasin E. Each point represents the average of triplicate plates.

that D-glucose can displace cytochalasin B to some extent from the high-affinity binding sites [3] while others have reported that D-glucose does not displace cytochalasin B from these sites [14]. In the data in Table I, we show that in the intact hepatocytes, D-glucose even at a concentration of 0.5 M does not displace cytochalasin B.

Cytochalasin B inhibition of 3-O-methyl-D-glucose transport in intact cells. Although D-glucose did not inhibit cytochalasin B binding to intact hepatocytes, the drug does inhibit sugar uptake in these cells (Fig. 4). It has been shown that 3-*O*-methyl-D-glucose and D-glucose are transported by the same mechanism in several cell types [7,18]. The transport of the non-metabolizable sugar, 3-*O*-methyl-D-glucose, in rat liver hepatocytes has previously been characterized [18]. It was shown that both uptake and efflux of the sugar occur very rapidly, since the intracellular concentration rapidly approaches equilibrium. As evidenced in Figs. 4 and 5, cytochalasin B at a concentration of 10^{-5} M has a significant inhibitory effect on 3-*O*-methyl-D-[^{14}C]glucose transport in the intact cell.

Effect of cytochalasin E on cytochalasin B binding and glucose transport. Cytochalasin E is an analogue of cytochalasin B which is a competitive inhibitor of cytochalasin B at those binding sites on the plasma membrane which appear not to be involved with the glucose carrier [9]. We found that cytochalasin E has no inhibitory effect on 3-*O*-methyl-D-glucose transport into intact cells or sugar efflux (unpublished data). Cytochalasin E did not displace cytochalasin B ($5 \cdot 10^{-7}$ M) from the intact cells until high levels of cytochalasin E were reached (Fig. 6). This is consistent with the assertion that cytochalasin E does not displace cytochalasin B from the 'high-affinity' sites which may represent the glucose-transport system.

Discussion

Cytochalasin B is a potent inhibitor of hexose uptake, cell division and locomotion [2—11]. The mechanism through which the drug causes these effects appears to be multifocal, since the dose-response curves differ significantly and the effects are extremely diverse. This implies that the drug binds to several receptors and, in fact, several investigators have demonstrated that cytochalasin B does have multiple binding sites. Spudich and Lin [27] showed that cytochalasin B had some interaction with actin and the actin-myosin complex, and later proposed [28] that in the red blood cell membrane the majority of the high-affinity cytochalasin B-binding sites were associated with the glucose-transport mechanism. Kasahara and Hinkle [29] substantiated this proposal in reconstituted liposomes containing erythrocyte protein which bound cytochalasin B and found that glucose transport was inhibited. Jung and Ranpal [9] have demonstrated three separate, saturable cytochalasin B-binding sites in human erythrocyte ghosts. All three were high-affinity sites but apparently only two were associated with glucose transport. Others have found that there seem to be high-affinity cytochalasin B-binding sites which are not associated with hexose transport. These have been reported in red cells [7,30] and in the plasma membrane of adipose cells [19,31].

Cytochalasin B is an effective inhibitor of hexose transport in rat liver

hepatocytes (Figs. 3 and 4). This also has been observed in several cell systems [2—10,13,19,28,35—40], with the exact results varying significantly between studies. It is possible that these differences could be due to the use of numerous different cell types, as well as variation in technique. The reported K_i in the above studies for cytochalasin B inhibition of hexose transport was very similar to the apparent dissociation constant for the high-affinity cytochalasin B-binding site of about 10^{-7} M found in this study. In fat cells [19], human red blood cells [9] and human fibroblasts [41], similar observations have been made. Due to this agreement, the assumption can be supported that at least some of these high-affinity binding sites have interactions resulting in the inhibition of hexose transport.

As apparent from the data contained in Table I, D-glucose was not able to inhibit cytochalasin B binding over wide concentration ranges of both molecules in the cell studied. However, since cytochalasin B did inhibit hexose transport in the hepatocytes, we assume that it did so through a noncompetitive mechanism. Others have reported that D-glucose inhibits high-affinity cytochalasin B binding nearly completely [28], partially [9] or not at all [19]. This divergence of results may be owing to the possibility that the glucose-transport system differs slightly from cell type to cell type. There is considerable controversy as to the kinetic mechanism for cytochalasin B inhibition of sugar transport. Findings of noncompetitive modes of inhibition, as evidenced here, have been demonstrated in fat cells [19,42—44], rat liver membranes [14], normal and transformed mouse cells [45], normal human fibroblasts [41] and rat hepatoma cells [46]. Alternatively, competitive inhibition, with D-glucose displacing cytochalasin B, has also been demonstrated in several cell types [3,47]. To complicate further the question, competitive and noncompetitive inhibition have been reported for the same cell system [46,47], and have varied depending on the precise experimental protocol [10].

Cytochalasin E appears to inhibit cytochalasin B binding to sites not involved in hexose transport [9]. We tested this analogue to see its effects on cytochalasin B binding and glucose transport. It was found that cytochalasin E has no effect on 3-*O*-methyl-D-glucose transport, and even at 10^{-5} M it inhibits only 25% of the cytochalasin B binding (cytochalasin B concentration was $5 \cdot 10^{-7}$ M). Since cytochalasin E at lower concentration has no effect on cytochalasin B binding, it would appear that the high-affinity sites are involved in sugar transport. Precisely what percent of the high-affinity sites are involved in glucose transport is not known.

In an effort to characterize the K_D of cytochalasin B interaction with the plasma membranes of the intact rat liver hepatocyte, association as well as dissociation rate experiments were performed. However, the initial rates of both were found to be too rapid for meaningful interpretation. This finding agrees with that found by Lin and Spudich [28] and Czech [19]. Due to this difficulty, a Scatchard plot analysis [25] was performed, and although it is understood that information derived from Scatchard plots is not unequivocal, data which agreed with other kinetic constants within reasonable limits were derived. The binding pattern is representative of most simply, a two-site,

independent model, or, one class of negatively cooperating binding sites, which cannot be ruled out.

This amount of cytochalasin B which binds to the rat liver cells appears to be significantly greater than that which binds other cells previously studied. Based on the mean maximum binding capacity of the high-affinity cytochalasin B-binding sites derived from the Scatchard analysis, and the relationship of cell number to protein amount, we have calculated that there are approx. $2.9 \cdot 10^8$ glucose carriers per hepatocyte if we assume that all of the high-affinity sites represent carrier proteins. This greatly exceeds the value of $4 \cdot 10^5$ found in normal confluent chicken embryo fibroblasts [10], and when compared to transformed chicken fibroblasts which were found to contain 10-fold more glucose-specific cytochalasin B-binding sites [10], as well as red blood cell membranes [33,34], which were comparable at $4 \cdot 10^6$ carriers/cell. Thus, it appears that the rat liver parenchymal cells are a potenially rich source of the glucose-transport system for future work in isolating this important membrane component.

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