DIHYDROCYTOCHALASIN B

Biological Effects and Binding to 3T3 Cells

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ABSTRACT

Dihydrocytochalasin B (H_2CB) does not inhibit sugar uptake in BALB/c 3T3 cells. Excess H_2CB does not affect inhibition of sugar uptake by cytochalasin B (CB), indicating that it does not compete with CB for binding to high-affinity sites. As in the case of CB, H_2CB inhibits cytokinesis and changes the morphology of the cells. These results demonstrate that the effects of CB on sugar transport and on cell motility and morphology involve separate and independent sites. Comparison of the effects of H_2CB, CB, and cytochalasin D (CD) indicates that treatment of cells with any one of the compounds results in the same series of morphological changes; the cells undergo zeiosis and elongation at 2-4 μM CB and become arborized and rounded up at 10-50 μM CB. H_2CB is slightly less potent than CB, whereas CD is five to eight times more potent than CB in causing a given state of morphological change. These results indicate that the cytochalasin-induced changes in cell morphology are mediated by a specific site(s) which can distinguish the subtle differences in the structures of the three compounds. Competitive binding studies indicate that excess H_2CB displaces essentially all of the high-affinity bound [^{3}H]CB, but, at less than 5×10^{-5} M, H_2CB is not so efficient as unlabeled CB in the displacement reaction. In contrast, excess CD displaces up to 40% of the bound [^{3}H]CB. These results suggest that three different classes of high-affinity CB binding sites exist in 3T3 cells: sites related to sugar transport, sites related to cell motility and morphology, and sites with undetermined function.

KEY WORDS 3T3 cells · cytochalasin · hexose uptake · motility · morphology

Cytochalasin B (CB) has two distinct classes of effects on mammalian cells. At lower concentrations (0.1-10 μM) CB inhibits sugar transport (11, 12), and at higher concentrations (1.0-100 μM) it inhibits cell motility and drastically changes cell morphology (36).

We have previously reported that human erythrocytes have a class of high-affinity CB binding sites with a dissociation constant (K_D) $\cong$ 0.1 μM

(16, 17). Because a large portion of the high-affinity bound CB can be displaced by sugars which are substrates for the sugar transport system, we concluded that in these cells most of the high-affinity sites are closely associated with, if not identical to, the sugar binding site of the transport proteins (16, 18). Recently, there have been several reports on the lack of effect of sugars on high-affinity CB binding to various types of cells (1, 5, 9, 15, 22). It is possible that some of these sites are involved not in sugar transport, but in those aspects of cell motility and morphology that

are affected by low concentrations of CB (1–5 μM). This paper reports the effects of dihydrocytochalasin B (H_2CB), an analogue of CB which differs from the parent compound by the saturation of the C_2–C_3 double bond (see Fig. 1 for structure) on 3T3 cells. We found that H_2CB does not inhibit sugar transport but is quite similar to CB in affecting cell motility and morphology. We also related the results of these studies to the binding of H_2CB, measured by competitive displacement of bound [³H]CB, and concluded that 3T3 cells probably contain a minimum of three different types of high-affinity CB binding sites.

MATERIALS AND METHODS

Materials

H_2CB, the product of the reduction of CB by $NaBH_4$, was prepared in our laboratory as described in detail elsewhere (15). Unlabeled CB and cytochalasin D (CD) (see Fig. 1 for structures) were purchased from Aldrich Chemical Company, Milwaukee, Wis. [³H]CB (7.6 Ci/mmol) prepared by the method of Lin et al. (14) was purchased from New England Nuclear, Boston Mass.

Stock solutions of H_2CB, CB, CD, and [³H]CB were prepared in dimethylsulfoxide (DMSO) and stored at 4°C. Control conditions included equivalent amounts of DMSO; final concentration of DMSO was always less than 2%.

Unlabeled 2-deoxy-D-glucose (DOG) was obtained from Sigma Chemical Co., St. Louis, Mo., [1-³H]DOG

(19 Ci/mmol) from Amersham/Searle Corp., Arlington Heights, Ill., and [¹⁴C]inulin (2.55 mCi/g) from New England Nuclear.

Cell Culture

BALB/c 3T3 and SV3T3 cells were used in these experiments. Cells were grown in Falcon plastic tissue culture dishes in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y., H-16) supplemented with 10% calf serum (DME), in an incubator containing humidified 5% CO_2 atmosphere. Cells were removed from the dishes for passage or experiments with 0.1% trypsin (Difco Laboratories, Detroit, Mich., 1:250), in Dulbecco's phosphate-buffered saline without divalent cations (PBS). We have previously shown that cells removed from dishes with trypsin bind CB to the same extent as those removed with EDTA (1). After an equal volume of DME was added to inactivate the trypsin, the suspension was either used for the next passage or centrifuged for 5 min at 250 g, and the cells were resuspended in PBS for binding assays. The number of cells per milliliter of suspension was determined with a hemacytometer. Protein content was determined by the Hartree modification of the Lowry method (6).

Sugar Uptake Assay

The procedure used was essentially that described by Sefton and Rubin for chick embryo fibroblasts (28). Approx. 1 × 10⁵ cells in 2 ml of DME were added to each 35-mm culture dish the day before the assay.
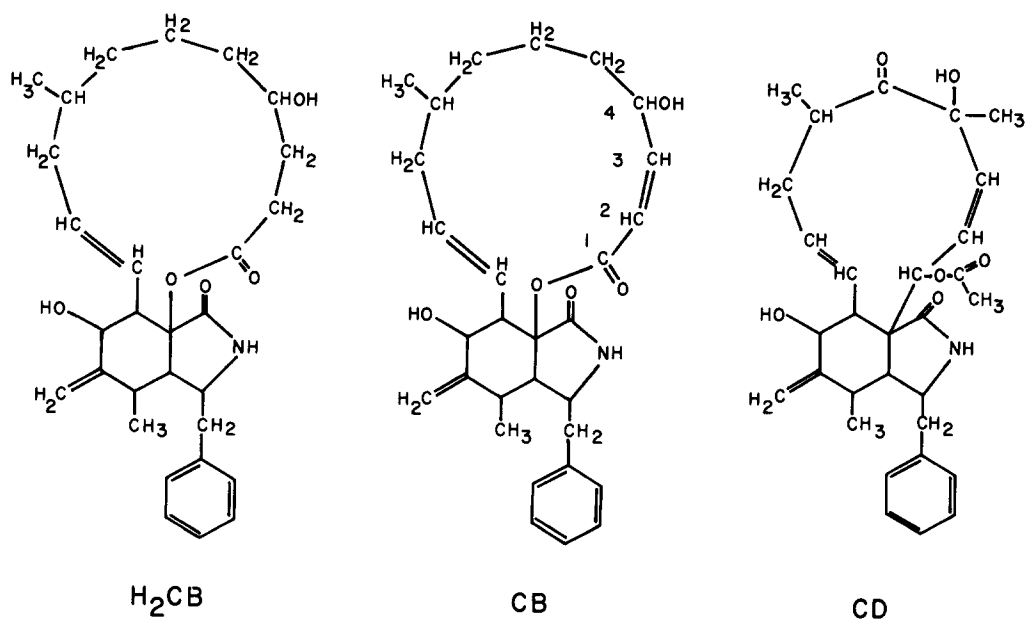

FIGURE 1   Chemical structures of the cytochalasins.

DME was removed by aspiration and the cells were washed twice with 3 ml of 37°C Hanks' balanced salt solution without glucose, buffered to pH 7.3 with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES-Hanks). To start the assay, one milliliter of HEPES-Hanks containing 0.3 μM [³H]DOG (1 μCi) and a designated amount of H_2CB, CB, or CD was added to each dish, and the cells were maintained at 37°C. After the specified incubation period, medium was removed by aspiration, and the cells were washed three times with 3 ml of ice-cold HEPES-Hanks. The entire washing procedure took ~20 s. The cell layer was digested in 1 ml of 1 N NaOH for 1 h at room temperature. A 0.2-ml aliquot of the digest was neutralized with acetic acid and the amount of [³H]DOG was determined by scintillation counting. Duplicate 0.3-ml aliquots of the digest were used for determination of protein content.

Photomicrography

Cells were seeded at ~3-4 × 10⁴ cells/35-mm culture dish in DME. The next day, DME was removed by aspiration, and 2 ml of warmed HEPES-Hanks containing H_2CB, CB, or CD was added to the cells. After incubating for 1 h at 37°C, the cells were photographed with a Nikon MS inverted microscope equipped with an AFM photographic system, using Kodak SO-410 film.

Inhibition of Cytokinesis

Cells were seeded at 1 × 10⁴ cells/35-mm culture dish in DME. The following day, DME was removed and fresh DME containing H_2CB or CB was added to the cells. After 48 h, the cells were examined with an inverted microscope equipped with phase-contrast optics. All the cells in random fields were scored as mononucleated, multinucleated, or indistinguishable (usually rounded and phase bright) until at least 100 cells had been counted.

CB Binding Assay

Binding of [³H]CB to cells was determined with a centrifugation assay as previously described (1, 14). Briefly, cells were incubated in buffer containing [³H]CB in the presence or absence of unlabeled H_2CB, CB, or CD, for 20 min, at room temperature. The cells were then removed from the assay medium by centrifugation, and the amount of [³H]CB associated with the cell pellet and the concentration of free [³H]CB in the supernate were determined by scintillation counting. Bound [³H]CB was calculated from radioactivity in the cell pellet after subtracting trapped counts as measured in [¹⁴C]inulin controls. High-affinity binding sites have been defined as those with $K_D \leq 10^{-5}$ M (14). In this paper, sites which are saturable with 10^{-4} M CB are referred to as high-affinity sites.

RESULTS

Effect of Cytochalasins on Sugar Uptake

One of the difficulties in studying the molecular basis of action of CB is the problem of separating the effect of the drug on sugar transport from the effects on cell motility and morphology. We have previously shown that H_2CB does not efficiently displace high-affinity bound [³H]CB from human erythrocyte ghosts (16). Because most of the high-affinity CB binding sites in these cells are related to sugar transport, this result suggests that H_2CB may have little or no effect on the uptake[1] of sugars into cells. We tested this possibility by examining the effects of CB and H_2CB on the uptake of DOG, an analog of D-glucose which is transported and phosphorylated but not further metabolized, by 3T3 cells. Fig. 2 illustrates that uptake in the presence and absence of cytochalasins was linear in both normal and virus-transformed cells for at least 15 min. The difference in the effects of the two cytochalasins was quite striking; 1 μM CB inhibited DOG uptake by 50% whereas an equal concentration of H_2CB produced no detectable effect. At 10 μM, CB inhibited uptake by 90% and H_2CB inhibited uptake by only 10%. Preincubation of the cells in the presence of the cytochalasins for 15 min gave similar results. In similar experiments, we found that CD at 1.0 and 10.0 μM had no detectable effect on uptake of DOG in these cells (data not presented). Although the rate of DOG uptake in virus-transformed cells was twice that of the normal cells, the effects of the cytochalasins were the same in both types of cells.

If H_2CB were binding to CB binding sites related to sugar transport, but did not affect the transport process, then excess H_2CB would diminish the inhibitory effects of CB on uptake. Fig. 3 illustrates that this was not the case; the addition of 10.0 μM H_2CB did not change the extent of uptake inhibition by 0.1-10.0 μM CB.

Effect of Cytochalasins on Cytokinesis

CB affects many different forms of cell motility, including membrane ruffling, cell locomotion, and cytokinesis (36). The inhibition of cytokinesis by CB, one of the best characterized effects of the drug, is related to the disruption of actin-containing microfilaments of the contractile ring

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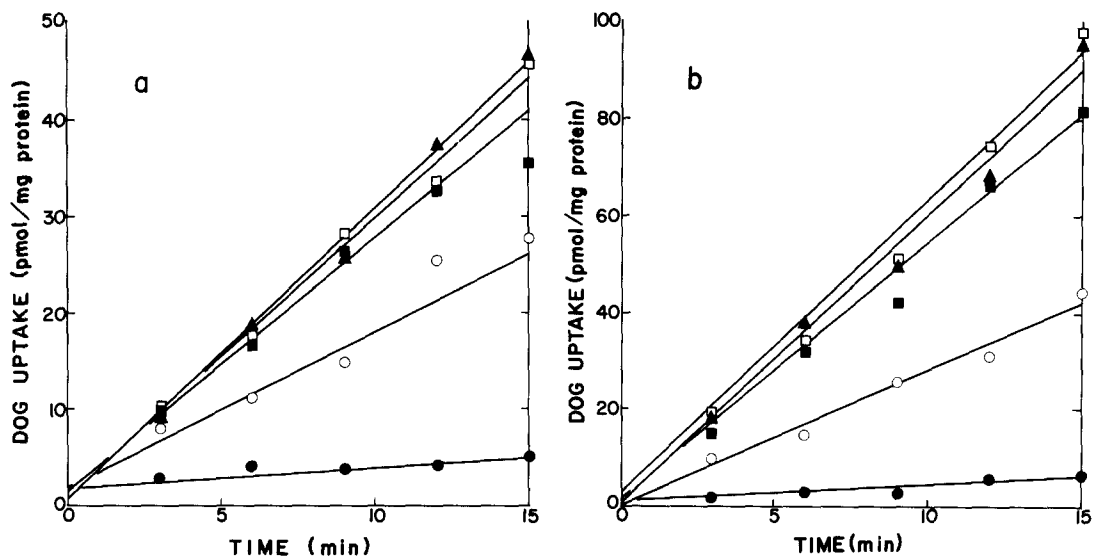[1] Uptake of DOG is a function of both transport and phosphorylation of the sugar.

FIGURE 2 Effect of cytochalasins on sugar uptake into 3T3 cells (a) and SV3T3 cells (b) as a function of time. Uptake of [³H]DOG was measured in the presence of DMSO (▲), 1 μM H₂CB (□), 10 μM H₂CB (■), 1 μM CB (○), or 10 μM CB (●). Lines are least square plots of data points.
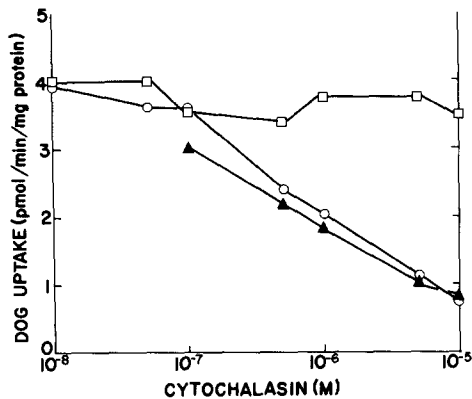

FIGURE 3 Uptake of [³H]DOG by 3T3 cells as a function of cytochalasin concentration. Uptake was measured at 10 min, in the presence of H₂CB (□), CB (○), or 10^{-5} M H₂CB plus the indicated concentration of CB (▲).

located at the cleavage furrow (27, 36). We have used this system to test the effect of H₂CB on motility of 3T3 cells. We found that CB produced multinucleated cells at a slightly lower concentration than H₂CB; however, once the threshold concentration was reached, the two drugs had similar effects on cytokinesis (Fig. 4). At concentrations higher than 10.0 μM, the large number of rounded cells made it impossible to accurately measure the percent of multinucleated cells. As reported by Kelly and Sambrook (10), normal
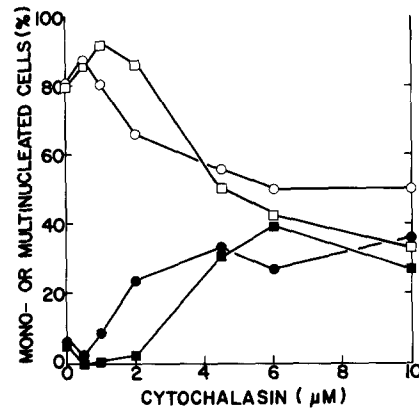

FIGURE 4 Inhibition of cytokinesis in 3T3 cells by cytochalasins. Cells incubated in the presence of H₂CB (squares) or CB (circles) for 48 h were scored as mononucleated (open symbols) or multinucleated (closed symbols). Totals do not equal 100% because in each experiment there were cells with an indistinguishable number of nuclei due to cell rounding in response to the presence of cytochalasin or due to mitosis.

3T3 cells appear to go through only one cycle of division in the presence of cytochalasin; very few cells had three or more nuclei.

Effects of Cytochalasins on Cell Morphology

3T3 cells are usually flat and thinly spread on

plastic or glass substrates, and, when examined with phase-contrast microscopy, appear phase-dark, with distinct nuclei and other organelles (Fig. 5a). We have compared the morphological responses of 3T3 cells to H₂CB, CB, and CD. We found that increasing concentrations of all three cytochalasins induced the same sequence of distinct morphological changes in these cells. H₂CB was very similar to CB in potency whereas CD was five to eight times more effective. The first distinct change was the elongation of the cell and the appearance of zeiotic knobs (see reference 20 for description) at the cell margins, seen with 4 μM H₂CB, 2 μM CB, and 0.5 μM CD (Fig. 5 b-d). 6 μM H₂CB or 4 μM CB induced coales-

cence of zeiotic knobs into discrete regions, sometimes accompanied by nuclear extrusion (Fig. 5e and f). Higher concentrations of cytochalasins (8 μM H₂CB or CB; 1 μM CD) caused the cells to retract most of the cytoplasm towards the center of the cell, leaving an arborized pattern of cell processes attached to the substrate (Fig. 6a-c). The final stage of morphological change was cell rounding (10 μM H₂CB or CB; 2 μM CD); the cells were phase-bright spheres firmly attached to the substrate by fine cytoplasmic processes (Fig. 6d-f). The effects of the three cytochalasins appeared to be additive. Cells treated with 2 μM CB plus 4 μM H₂CB exhibited nuclear extrusion similar to cells in 4 μM CB; cells treated with 2
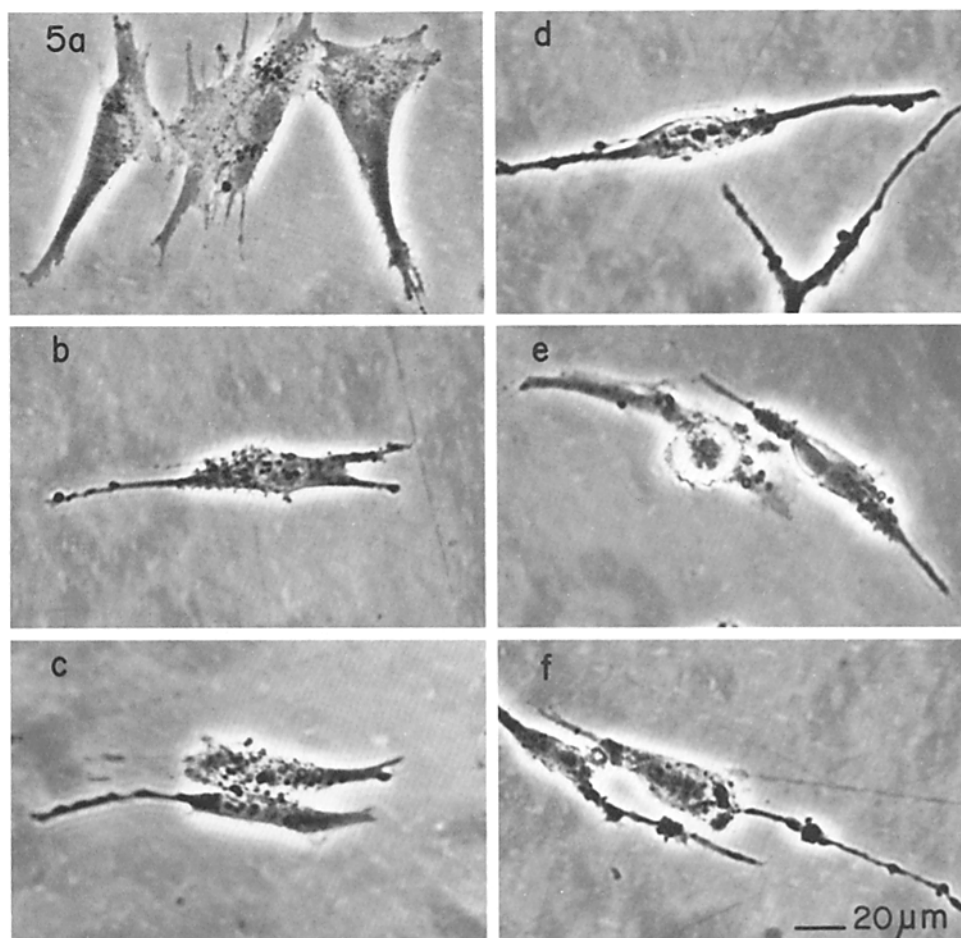

FIGURE 5 Effects of lower concentrations of cytochalasins on morphology of 3T3 cells. The cells were incubated at 37°C for 1 h in the presence of DMSO or cytochalasin. (a) DMSO control. Cells exhibited zeiotic knobs with (b) 2 μM CB, (c) 4 μM H₂CB, and (d) 0.5 μM CD. More extensive zeiosis accompanied by nuclear extrusion was seen with (e) 4 μM CB and (f) 6 μM H₂CB. × 350.
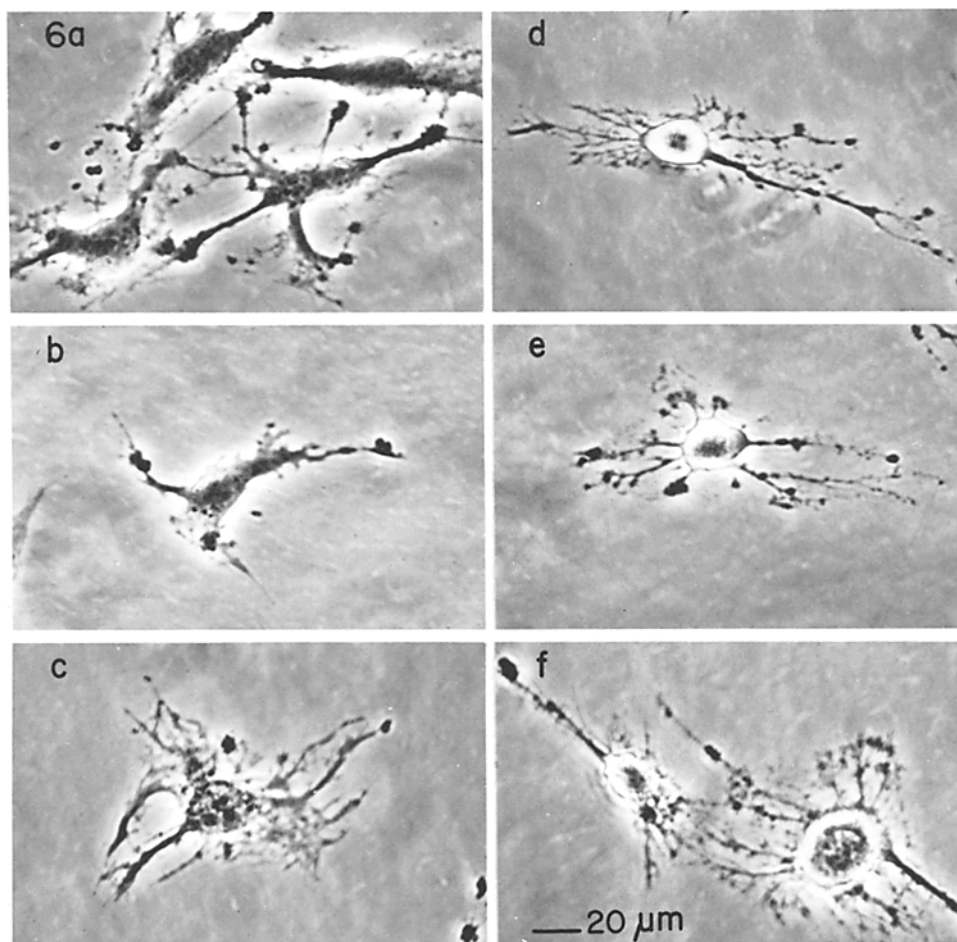
FIGURE 6 Effects of higher concentrations of cytochalasins on morphology of 3T3 cells. After incubation at 37°C for 1 h, arborization was seen with (a) 8 μM CB, (b) 8 μM H$_2$CB, and (c) 1 μM CD. Cells were rounded in (d) 10 μM H$_2$CB, (e) 10 μM CB, and (f) 2 μM CD. × 350.

μM CB plus 6 μM H$_2$CB were arborized similar to cells in 8 μM CB (Fig. 7a and b). Similar results were seen with the addition of CB to CD (Fig. 7c and d), confirming the findings of Tannenbaum et al. (33).

Competitive Binding of Cytochalasins to Cells

3T3 cells have high-affinity binding sites for [^{3}H]CB (1). We have indirectly studied the binding of unlabeled H$_2$CB to these sites by measuring the displacement of bound [^{3}H]CB by H$_2$CB. We first demonstrated that bound [^{3}H]CB is stoichiometrically displaced by unlabeled CB, showing that the two species have similar affinities for binding sites in 3T3 cells. As shown in Fig. 8, the

number of CB molecules bound at a given concentration of free CB was essentially the same regardless of whether undiluted [^{3}H]CB or labeled material diluted fourfold or eightfold with unlabeled CB was used (see reference 14 for discussion of this type of isotope dilution experiment). Thus, the displacement of bound [^{3}H]CB is a valid indication of the binding of unlabeled cytochalasin to these cells.

Binding of 0.008–8.0 μM [^{3}H]CB was inhibited to the same extent by the addition of a high concentration (50 μM) of H$_2$CB or CB (Fig. 9a and b). Scatchard plot analysis of the binding data confirms our previous report that there are high- and low-affinity CB binding sites in 3T3 cells (Fig. 9c). [^{3}H]CB which can be displaced by
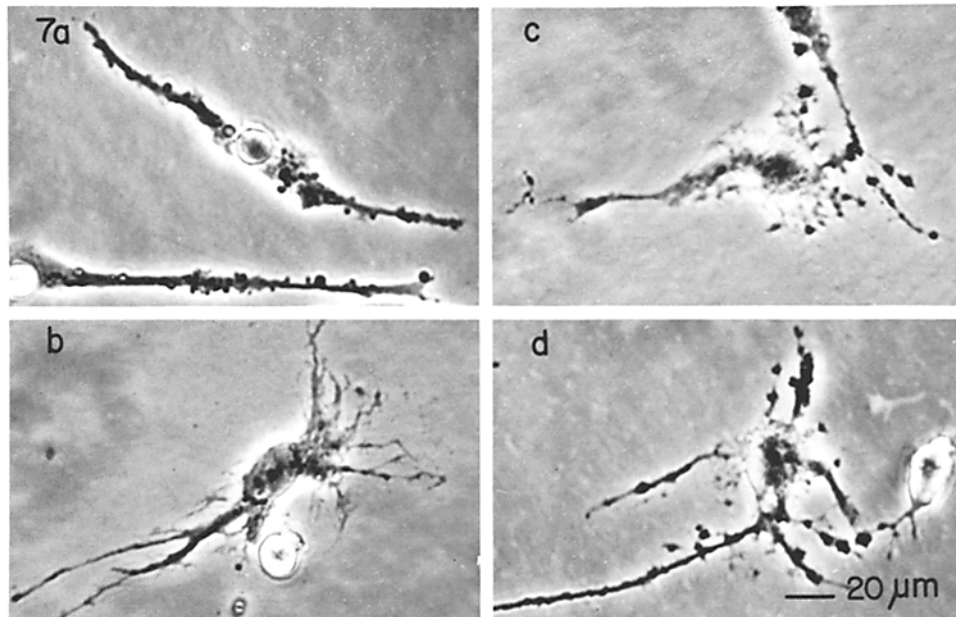
FIGURE 7 Additive effects of cytochalasins on 3T3 cell morphology. 2 μM CB alone produced zeiosis (see Fig. 5c); cells exhibited more severe zeiosis accompanied by nuclear extrusion in 2 μM CB plus 4 μM H$_2$CB (a), and arborized in 2 μM CB plus 6 μM H$_2$CB (b). 0.5 μM CD produced zeiosis (see Fig. 4d); cells arborized in 0.5 μM CD plus 2.0 μM CB (c) and rounded up in 0.5 μM CD plus 4 μM CB (d). × 350.
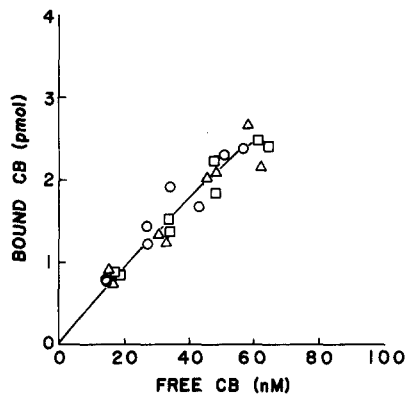

FIGURE 8 Binding of [³H]CB of different specific activities to 3T3 cells. Samples containing 4 × 10⁵ cells were incubated for 20 min in various concentrations of labeled CB using undiluted [³H]CB (O), [³H]CB diluted fourfold with unlabeled CB (△), and [³H]CB diluted eightfold with unlabeled CB (□).

excess unlabeled CB is referred to as high-affinity bound CB.

Experiments in which a wide range of cytochalasin concentrations was used were performed to compare the relative effectiveness of H$_2$CB, CB, and CD in displacing bound [³H]CB (Fig. 10).

Excess H$_2$CB displaced bound [³H]CB to the same extent as CB, but was not so effective as CB at lower concentrations. In contrast, CD displaced only ~40% of high-affinity bound [³H]CB, even at very high concentrations; the data were insufficient to determine whether CD was as effective as CB for displacing that portion of the bound [³H]CB.

DISCUSSION

We have found that H$_2$CB, derived from CB by the saturation of a single carbon-carbon double bond, had little or no effect on sugar uptake in 3T3 cells. Excess H$_2$CB did not affect inhibition of sugar transport by CB, indicating that H$_2$CB does not compete with CB for binding to transport-related sites. The slight inhibition of DOG uptake measured at 10 μM H$_2$CB was either due to trace amounts (<1%) of contaminating CB undetectable by chemical analysis, or to the low-affinity binding of small amounts of H$_2$CB to transport-related sites. Our results are in agreement with the finding that H$_2$CB does not inhibit D-glucose transport in human erythrocytes (13).

Our observation that sugar transport-related CB binding sites have extremely rigid require-
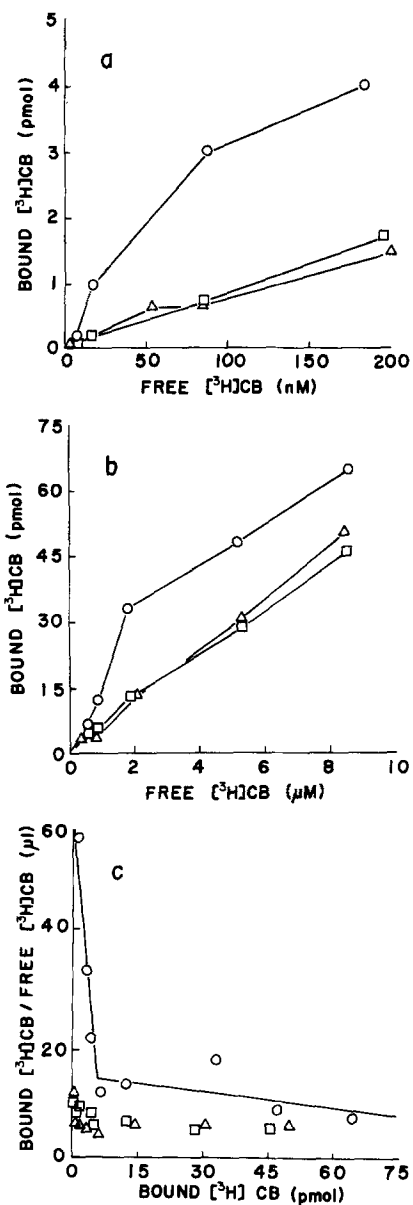
FIGURE 9 Inhibition of binding of [^{3}H]CB to 3T3 cells by excess unlabeled H_2CB or CB. Samples containing 2 × 10^5 cells were incubated for 20 min in various concentrations of [^{3}H]CB in the presence of DMSO (O), 50 μM H_2CB (□), or 50 μM CB (△). (a) Binding at low concentrations of [^{3}H]CB (0.008–0.18 μM). (b) Binding at high concentrations of [^{3}H]CB (0.5–8.0 μM). (c) Scatchard plot analysis of data shown in (a) and (b).

ments for ligand structure is consistent with the predictions made by Taylor and Gagneja (35) that the spatial disposition of the four oxygen

atoms in CB is essential for its ability to inhibit the transport process. The saturation of the C_2–C_3 double bond apparently results in enough movement of the C_4 oxygen atom that the resulting compound (H_2CB) is no longer able to bind with high affinity to the transport-related sites. This conjecture is also in agreement with our earlier finding that H_2CB is a weak competitor for high-affinity CB binding sites which are related to sugar transport in human erythrocytes (15, 16).

Our comparative studies on the effect of CB on sugar transport in normal and transformed BALB/c 3T3 cells confirm earlier reports that transformed cells have a higher rate of sugar transport (for review, see reference 7). The observation that CB inhibited transport to a similar extent in both types of cells indicates that viral transformation did not alter the CB binding properties of the transport system. The CB concentration necessary to achieve 50% inhibition of transport was ~1 μM, a value similar to those determined in a variety of cultured mammalian cells (23), but higher than those reported for human erythrocytes (2, 34, 35) and chick embryo fibroblasts (11, 12).

Consistent with studies on a wide variety of motile systems (13), we have found that H_2CB affects cell motility and morphology in 3T3 cells in a manner almost indistinguishable from that of CB. The results, coupled with the finding that H_2CB does not significantly affect sugar transport, demonstrate clearly that the effects of CB on sugar transport and on cell motility and morphology involve separate and independent sites in 3T3 cells. The data presented here rule out the possibilities suggested by other investigators that the two types of CB effects are mediated by a single membrane receptor (2) or are caused by the nonspecific partitioning of the drug into the lipid phase of the plasma membrane (8, 19, 24, 29).

The effects of CB on the motility and morphology of 3T3 cells are similar to those described by other investigators (3, 4, 10, 26). Detailed comparison of the concentration dependence of the effects of H_2CB, CB, and CD leads to the following conclusions on the mode of action of the cytochalasins. First, treatment of the cells with any one of the three drugs resulted in the same morphological changes; the cells exhibit elongation, zeiosis, arborization, and rounding up, depending on drug concentration. This, together with the observation that the effects of the differ-
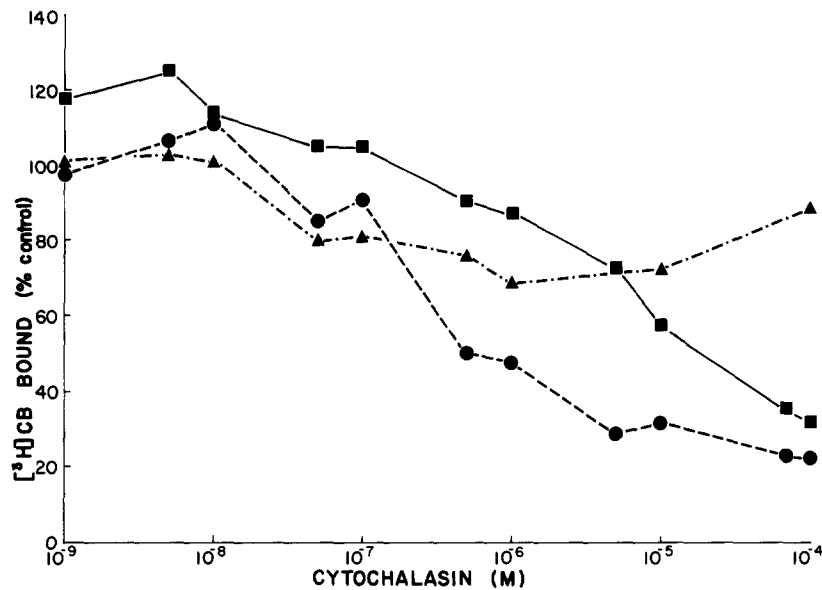
FIGURE 10 Concentration dependence of the inhibition of binding of [³H]CB to 3T3 cells by unlabeled cytochalasins. Samples containing 5 × 10⁵ cells were incubated in 0.05 μM [³H]CB and the indicated concentration of H_2CB (■), CB (●), or CD (▲). Results were calculated as percent of [³H]CB bound compared to controls containing DMSO.

ent drugs are roughly additive (confirming the findings of Tannenbaum et al. [33]), indicate that H_2CB, CB, and CD affect a common site(s) that is involved in cell motility and morphology. Second, we found that H_2CB is slightly less potent than CB and confirmed that CD is five to eight times more potent than CB (4) in causing a given state of morphological change. This supports the view that the effects of the compounds are mediated by a specific site(s) which can distinguish the subtle differences in the structures of the cytochalasins. Third, the effects of the cytochalasins on cell morphology can þe divided into two classes: less drastic changes occurring at low drug concentrations, i.e., cell elongation and zeiosis, and gross changes occurring at high drug concentrations (cell arborization and rounding). The first type of effects, requiring about the same levels of CB necessary to inhibit sugar transport in these cells, may be mediated by high-affinity binding sites in the cell. These sites may be related to membrane-associated microfilament networks which have been shown by electron microscopy to be disrupted at low concentrations of cytochalasin (21). Consistent with this idea is the observation that treatment of isolated membrane preparations with agents known to depolymerize contractile proteins decreases high-affinity binding of

CB or CD (25, 32). The second type of effects, requiring 10–50 μM CB, could be caused by the low-affinity interaction of the drug with actin in a manner analogous to that observed in in vitro experiments (18, 30, 31). Studies on the effect of CB and H_2CB on cytokinesis performed over 48 h were, by necessity, carried out in growth medium supplemented with 10% calf serum. Because serum has been shown to decrease binding of CB to cells (14), the concentration dependence of this effect cannot be directly compared to studies on cell motility and morphology performed in serum-free media.

We have previously reported that human erythrocytes have two classes of high-affinity CB binding sites. The first class of sites (3 × 10⁵ per cell), which bind CB and D-glucose but not H_2CB, are located on the sugar transport proteins in the membrane (16, 17). The second class of sites (5 × 10⁴ per cell), which bind CB and H_2CB but not D-glucose, are not related to the sugar transport process (15). The biological function of these sites has not yet been determined. Our studies on the competitive binding of H_2CB, CB, and CD to 3T3 cells indicate that the makeup of the high-affinity CB binding sites in these cells is quite different from that of human erythrocytes. We found that an excess of H_2CB displaced essentially

all high-affinity bound [³H]CB, but at any given concentration H₂CB is not so effective as unlabeled CB in the displacement reaction. In contrast, an excess of CD displaced a maximum of 40% of the high-affinity bound [³H]CB; the relative effectiveness of this compound in the displacement reaction has not been determined. As previously described (1), D-glucose has no measurable effect on the binding of [³H]CB to 3T3 cells.

On the basis of our knowledge of the biological effects of H₂CB, CB, and CD on 3T3 cells, we can tentatively classify the high-affinity CB binding sites in these cells as follows: First, there is a class of sites which are related to the inhibitory effect of CB on sugar transport. If these sites bind CB and D-glucose competitively, then the number of these sites must be immeasurably small when compared to the total number of high-affinity sites in these cells. On the other hand, if these sites do not bind D-glucose competitively, i.e., CB binds to a part of the transport system which is not the sugar binding site, then the number of these sites cannot exceed 45% of all of the high-affinity CB binding sites in the cells. This estimate is based on the observation that 10 μM H₂CB, which had a minimal effect on sugar transport, inhibited high-affinity [³H]CB binding by 55%. Second, there is a class of high-affinity CB binding sites which are likely to be related to some, if not all, of the effects of the cytochalasins on cell motility and morphology; these are sites which bind H₂CB and CB with similar affinity. Measurements of the maximum displacement of [³H]CB by excess CD indicated that these sites make up ~40% of the total number of high-affinity CB binding sites in the cells. Finally, there may be a third class of sites which do not bind CD, but bind CB with high affinity and H₂CB with lower affinity. The presence of such sites would explain the observation that the amount of [³H]CB displaced by H₂CB at <5 × 10⁻⁵ M is significantly less than that displaced by the same concentration of unlabeled CB. The number of these sites per cell and the biological function of this proposed class of sites are unknown at this time.

High-affinity sites which bind cytochalasins but not D-glucose have been described in a number of cells. CB binding to fat cells (5) and Novikoff cells (22) is not inhibited by D-glucose; a large portion of CB binding to Novikoff cells is inhibited by an excess of other cytochalasins. Human and bovine erythrocytes have a relatively small number of sites which bind CB, H₂CB, and CD, but

not D-glucose (15). It is possible that these sites are related to sites in 3T3 cells which are involved in cell motility and morphology. Tannenbaum et al. (33) proposed that there are two classes of binding sites for CD in HEp2 cells: sites which bind CD with high affinity but do not bind CB, and sites which bind CD and CB with low affinity. Both of these classes of sites are thought to be involved in the effects of CD on cell motility and morphology. The results of our study on 3T3 cells do not support their proposal, as we have found that CB and CD share at least some high-affinity sites. Moreover, the observation that CB and CD produced the same morphological changes in these cells argues against the presence of high-affinity sites which bind only CD. The apparent discrepancy between our results and those reported by Tannenbaum et al. (33) may be due to differences in cell type and experimental conditions used in the binding assays.

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