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7-Acetylcytochalasin B: Differential Effects on Sugar Transport and Cell Motility

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Cytochalasin B (CB) is a potent inhibitor of sugar transport and cell motility in animal cells. We have synthesized and characterized the CB derivative 7-acetylcytochalasin B (CBAc) and have found that it has differential effects on transport and motile processes in fibroblasts. The derivative inhibited sugar transport in human red cells, 3T3 cells, and chicken embryo fibroblasts at micromolar concentrations, although it was less potent than its parent compound. Unlike CB, which causes fibroblasts to round up and arborize at less than 10 μM, CBAc had no effect on fibroblast morphology and membrane ruffling at concentrations as high as 90 μM. Competitive binding experiments using [^{3}H]CB showed that the affinity of CBAc for sites related to sugar transport in the red cell membrane is about one-fourth of that of CB. In contrast, similar experiments using [^{3}H] dihydrocytochalasin B (a derivative which inhibits cell motility but not sugar transport) showed that the affinity of CBAc for sites associated with red cell spectrin and actin is only about 1/20 of that of dihydrocytochalasin B. This study demonstrates that acetylation of the C-7 hydroxyl group of CB reduces its effect on cell morphology and motility much more than its ability to inhibit sugar transport. This observation, together with our earlier work with dihydrocytochalasin B, establishes that the pharmacologic effects of CB on fibroblasts result from the binding of the drug to two distinct classes of receptors and that these receptors interact with different parts of the cytochalasin molecule.

Key words: cytochalasin B derivative, cell motility, sugar transport

Cytochalasin B (CB) (the numbering of the carbon atoms is according to Tanenbaum [1]) exerts two major classes of effects on animal cells. At 0.1–1 μM, the drug inhibits facilitated diffusion of sugars into mammalian and avian cells [1]. At 1–100 μM, the drug affects cell morphology and inhibits many types of motile processes, such as cell locomotion, membrane ruffling, and cytokinesis [1].

Abbreviations used: CB, cytochalasin B; H_2CB, dihydrocytochalasin B; CBAc, 7-acetylcytochalasin B; $CBAc_2$, 7, 20-diacetylcytochalasin B; DME, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DOG, 2-deoxy-D-glucose.

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While the inhibitory effect of CB on sugar transport in the human red cell can be explained by the direct competition of the drug with sugars for binding to the transport protein [2–6], the molecular basis of cytochalasin action on cell motility is not well understood. Recently, we have shown that dihydrocytochalasin B (H_2CB), a derivative of CB which lacks the double bond between C-21 and C-22, is similar to its parent compound in its effects on cell morphology and motility, but is ineffective at blocking sugar transport [7, 8]. Utilizing the binding of [^{3}H]H_2CB as an assay, we were able to isolate from human red cells a class of high-affinity binding sites located in supramolecular complexes containing actin and spectrin [9, 10].

Cytochalasin binding to cultured fibroblasts is more complicated than to human red cells. Sugar transport in fibroblasts is a challenging system to cell biologists because it is regulated according to the physiologic state of the cell (eg, contact inhibition, starvation, viral transformation [11]). However, the use of CB as a probe in the fibroblast is complicated by the finding that there are at least three classes of CB-binding sites in this type of cell and that the majority of the sites are apparently unrelated to sugar transport [8]. Moreover, D-glucose displacement cannot be used as a reliable criterion to define transport-related sites because the sugar displaces bound CB from some cell types [12] but not from others [13, 14]. It appears, therefore, that the availability of cytochalasin derivatives which are specific only for sugar transport would greatly facilitate research in this area.

In this paper we report that the CB derivative, 7-acetylcytochalasin B (CBAc), has properties complementary to that of H_2CB. CBAc blocks sugar transport but has little effect on cell motility and morphology. These results corroborate previous results obtained with H_2CB, that the effects of CB on sugar transport and cell motility are mediated by different receptors [7, 8].

EXPERIMENTAL PROCEDURES

Materials

Unlabeled CB was purchased from Aldrich Chemical Co.; [^{3}H]CB (7.6 Ci/mmole) and [^{14}C] inulin (2.55 mCi/g) were bought from New England Nuclear. [^{3}H]-Labeled and unlabeled H_2CB were prepared from the corresponding forms of CB by reduction with $NaBH_4$, as previously described [3, 9]. Stock solutions of cytochalasins were made up in dimethyl sulfoxide (DMSO) and stored at 4°C. Unlabeled 2-deoxy-D-glucose (DOG) was obtained from Sigma Chemical Co. [1-^{3}H] DOG (19 Ci/mmole) was purchased from Amersham/Searle Corporation. Unless otherwise specified, human red cells were from blood generously donated by the Baltimore Red Cross Blood Center and used within two weeks after the blood was drawn. All reagents were of analytical grade and used without further purification.

Preparation and Characterization of CBAc

The synthesis of CBAc, presented schematically in Figure 1, follows the procedure of Masamune et al [15]. CB is first acetylated to 7,20-diacetylcytochalasin B ($CBAc_2$), which is then selectively hydrolyzed to CBAc. A typical preparation was done in the following manner. $CBAc_2$ was prepared by the slow addition of 100 μl of acetic anhydride to a stirred solution of 19.4 mg of CB in 200 μl of pyridine. The reaction was allowed to proceed overnight at room temperature, quenched by the addition of xylenes and the reagents were removed in vacuo. The product was purified by thin-layer chromatography (see below) to give 15.9 mg of $CBAc_2$. Selective hydrolysis was performed by adding 120

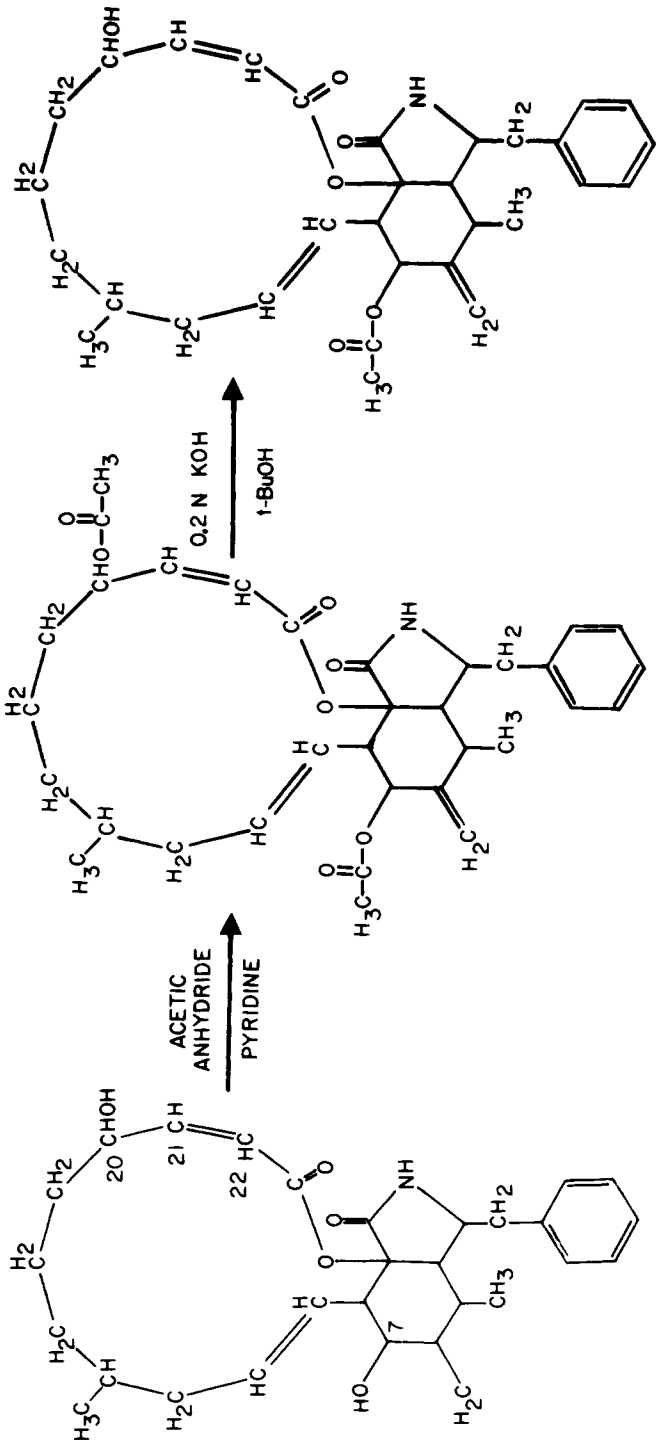

Fig. 1. Synthesis of 7-acetylcytochalasin B from cytochalasin B by the selective hydrolysis of 7,20-diacetylcytochalasin B. Numbering of carbon atoms is according to Tanenbaum [1].

μl of 0.2 N KOH to a solution of 12.7 mg of $CBAc_2$ in 750 μl of t-butanol and 250 μl of water at room temperature for 12 h. The progress of the hydrolysis reaction was followed by thin-layer chromatography. The crude reaction mixture was extracted into ether and washed with saturated NaCl, and the organic phase was dried in a stream of nitrogen. Purification by repeated thin-layer chromatography yielded 7 mg of CBAc.

Purity of the CBAc preparation was confirmed by reversed-phase high-pressure liquid chromatography, which gave a single peak of material. Low-resolution mass spectrometry of the purified product gave a molecular weight (Mr = 521.5) and a spectrum consistent with the structure of CBAc. That this procedure gave a CBAc preparation that contained less than 1% CB and $CBAc_2$ was also shown by using [^{3}H]CB as a tracer through the above synthesis and isolation procedure. Reduction of 7-acetylcytochalasin A with $NaBH_4$ to CBAc, using the procedure described for reduction of cytochalasin A to CB [16], gave a compound indistinguishable by thin-layer chromatography from the CBAc prepared by the hydrolysis method.

Thin-layer chromatography was performed on Analtech silica gel GF plates of 250 μm thickness. The plates were developed with chloroform/ethyl acetate (1:1). In this system the R_f values for CB, CBAc, and $CBAc_2$ are 0.4, 0.5, and 0.6, respectively. The compounds were revealed by charring with chromic acid/sulfuric acid/water (1:1:2). Material to be isolated was revealed by UV quenching of the fluorescent plates and eluted from scraped spots with the same solvent mixture as was used to develop the chromatogram.

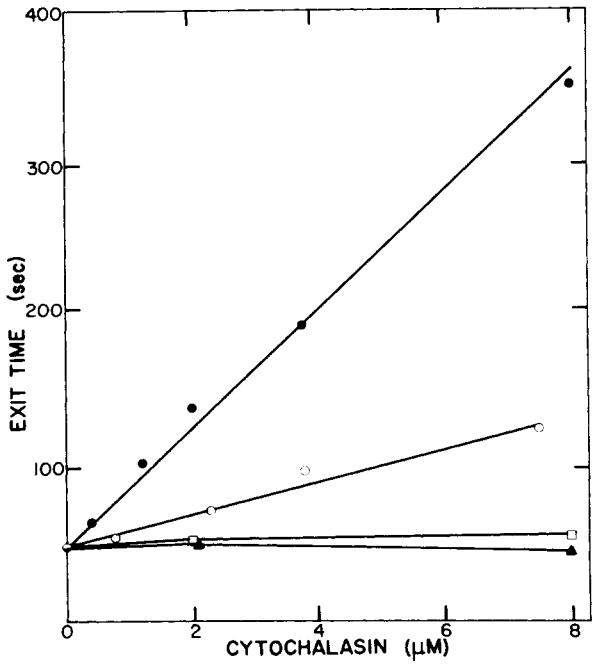

Fig. 2. Effect of cytochalasins on exit of D-glucose from preloaded human red cells. Washed red cells from freshly drawn blood were preloaded with sugar by incubation with 0.1 M D-glucose in 5 mM sodium phosphate buffer, pH 8, with 150 mM NaCl, for 30 min at 37°. Cells were then equilibrated to 25°. At zero time, 50 μl of the cell suspension (10% hematocrit) were added to a cuvette containing 2.5 ml of glucose-free buffer with the specified concentrations of CB (●), CBAc (○), H_2CB (□), $CBAc_2$ (▲), or 5 μl DMSO (⊖). The exit time, measured as described by Sen and Widdas [17], was monitored by following the change in optical density at 700 nm using a Cary 16 recording spectrophotometer.

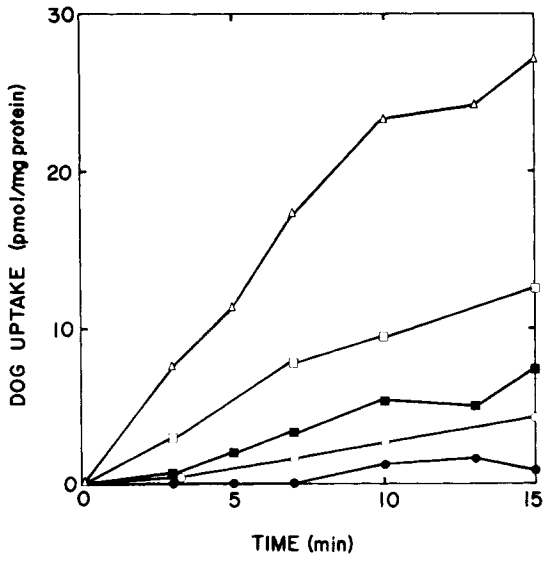

Fig. 3. Effect of cytochalasins on DOG uptake in 3T3 cells. Uptake was measured at 37° on approximately 10^5 cells, grown as monolayers on 35-mm dishes as described previously [8]. The assay medium contained Hanks' balanced salt solution without glucose, buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid to pH 7.3, 0.4 mM DOG, and 1.1 μCi of [^{3}H]DOG. Uptake was measured in the presence of 4 μM CBAc (□), 10 μM CBAc (■), 4 μM CB (○), 10 μM CB (●), and in the absence of any drug (△).

RESULTS

Effect of Cytochalasins on Sugar Transport

CB inhibits sugar transport at micromolar concentrations in mammalian and avian cells [1]. In the three cell types examined in this study, CBAc was also found to be an effective inhibitor of sugar transport, although not as potent as its parent compound.

The Sen and Widdas exit time method [17] was used to estimate the relative potency of several cytochalasins on sugar transport in human red cells. As shown in Figure 2, increasing the amount of CBAc in the assay medium caused the exit time of D-glucose to increase, although the effect was less than that of CB. In contrast, H_2CB and $CBAc_2$ had no effect on transport.

The effects of CB and CBAc on sugar uptake in 3T3 cells are shown in Figure 3. Based on the amount of sugar taken up by the cells at 10 min, CBAc at 4 and 10 μM inhibited the uptake process by 59 and 77%, respectively. In agreement with the results for the red cells, equivalent amounts of CB produced higher levels of inhibition (88 and 94%, respectively). Similar results were obtained with chicken embryo fibroblasts, cultured according to Vogt [18]: CBAc at 4 and 9 μM produced inhibition of 48 and 62%, respectively, as compared to to 88% produced by 4 μM CB.

Effect of Cytochalasins on Cell Morphology and Motility

3T3 cells show a distinct series of morphologic changes when treated with CB or H_2CB. With increasing concentrations of the drugs, the cells elongate, exhibit zeiosis, and finally round up and arborize [8]. As shown in Figure 4a–c, cells treated with 10 μM CB were fully

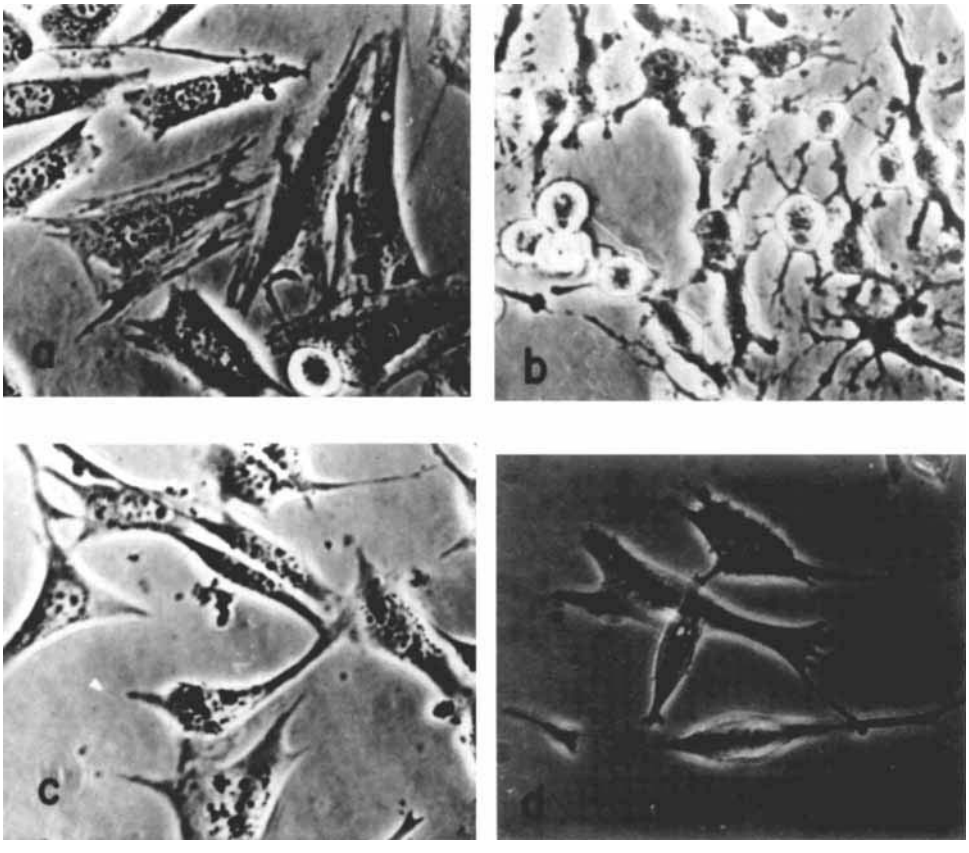

Fig. 4. Effects of cytochalasins on 3T3 cell morphology and motility. The cells were incubated at 37° for 1 h in the presence of DMSO or cytochalasin and then photographed [8]. a: DMSO-treated cells showing normal morphology. b: Cells rounded up and arborized in the presence of 10 μM CB. c: Cells showing normal morphology in the presence of 90 μM CBAc. d: Cells, in the presence of 90 μM CBAc, showing membrane ruffling (arrows) as judged by time-lapse video recording, performed with a Panasonic video recorder (model No. Nv-8030) and camera (model No. WV 1350) attached to a Nikon MS inverted scope. M × 300.

arborized, whereas cells treated with 90 μM CBAc had normal morphology. $CBAc_2$ at 90 μM also had no effect on cell morphology (not shown). However, when $CBAc_2$ was converted to CB by alkaline hydrolysis, it caused the cells to arborize, indicating that acetylation of the hydroxyl groups blocked the action of the cytochalasin. Results similar to that seen with 3T3 cells were obtained with chicken embryo fibroblasts. These cells were more sensitive to CB than 3T3 cells: 1–3 μM CB induced arborization whereas 50 μM CBAc did not significantly affect cell morphology even after 5 h.

Time-lapse video recording of membrane ruffling was used to study the effects of cytochalasins on motility of 3T3 cells. In control experiments (ie, cells were treated with DMSO), many areas of the membrane edges of the cells were seen to ruffle with an undulating movement when the video recording was played back at 108 × actual time; membrane ruffling was especially vigorous when cells were spreading. When cells were treated with 10 μM CB, membrane ruffling was inhibited within a few minutes. This inhibition was ac-

companied by the retraction of the cell margins towards the center of the cells, causing the cells to take on the arborized morphology as shown in Figure 4b. CBAc and $CBAc_2$, on the other hand, had no effect on membrane ruffling or on cell morphology at concentrations as high as 90 μM. This was true for both spreading cells and cells that had been plated the previous day. Figure 4d shows a photomicrograph of CBAc-treated cells which had been shown to exhibit membrane ruffling by time-lapse video recording.

Competitive Binding of Cytochalasins to Red Cell Membrane and Extract

The studies described in the preceding sections showed that CBAc inhibits sugar transport without affecting cell morphology and motility. The following experiments were designed to determine whether this kind of specificity reflects the affinity of CBAc for different types of high-affinity CB binding sites. The human red cell was used as a test system because the binding sites related to sugar transport can be separated from those related to motility and both types of sites have been previously characterized.

Membranes containing only transport-related CB binding sites were prepared by removal of motility-related sites by extraction of ghosts with EDTA at low ionic strength [9]. The relative affinity of CBAc compared with CB for the transport-related sites was estimated by measuring the displacement of [^{3}H]CB bound to the extracted membranes at various concentrations of the unlabeled forms of the two compounds. At 10^{-7} M [^{3}H]CB (a concentration approximately the same as the $K_{dissociation}$(Kd) of CB for transport-related sites [2]), 50% displacement of the labeled compound occurred at 0.24 μM CB compared with 1 μM CBAc (Fig. 5). This indicates that the affinity of the derivative for the transport-related sites is approximately 1/4 that of the parent compound. In a similar experiment,

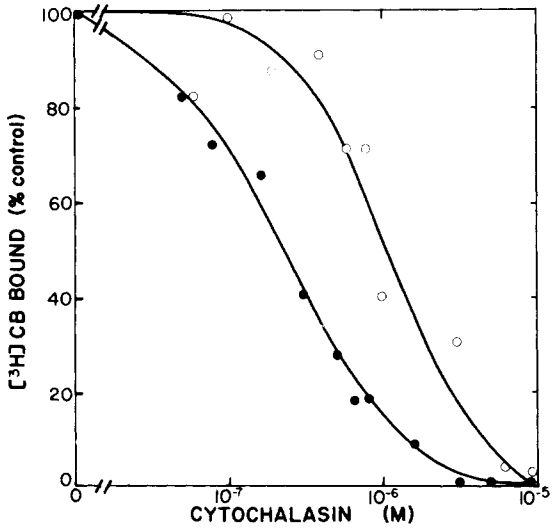

Fig. 5. Competitive binding of [^{3}H]CB and unlabeled cytochalasins to EDTA-extracted red cell membranes. EDTA-extracted membranes [9] derived from 5×10^8 cells were incubated in 600 μl of 5 mM sodium phosphate buffer, pH 8, containing the specified amounts of the unlabeled form of CB (•) or CBAc (○). After 10 min, [^{3}H]CB was added to the assay medium to a concentration of 10^{-7} M and the amount of labeled drug bound to the membrane was determined with the centrifugation assay [2] after another 10 min of incubation. High-affinity binding was defined as the portion of the total binding that was displaceable by 10^{-4} M unlabeled cytochalasin, after correcting for trapped counts measured in [^{14}C] inulin controls.

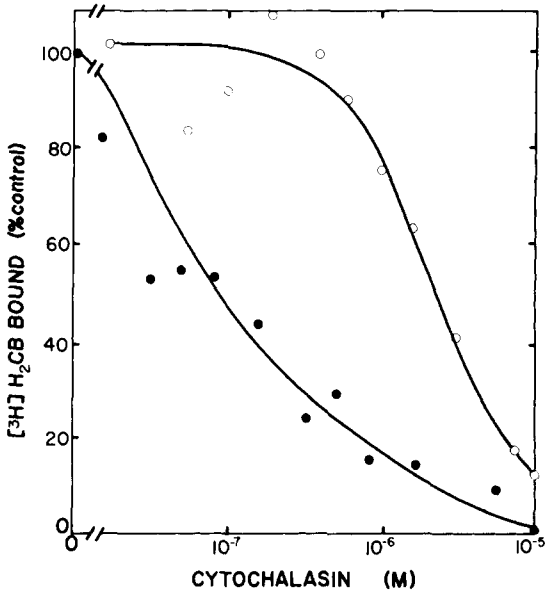

Fig. 6. Competitive binding of [^{3}H] H_2CB and unlabeled cytochalasins to sealed red cell ghosts. Sealed ghosts [9] prepared from 5×10^8 cells were incubated in 600 μl of 5 mM sodium phosphate buffer, pH 8, with 150 mM NaCl, containing the specified amounts of unlabeled H_2CB (•) or CBAc (○). After 10 min, [^{3}H] H_2CB was added to the assay medium to a concentration of 10^{-8} M and the amount of labeled drug bound to the membrane was determined with the centrifugation assay [2] after another 10 min of incubation.

the relative affinities of CBAc and H_2CB for motility-related sites were estimated by measuring displacement of [^{3}H] H_2CB from red cell ghosts by the two compounds. At 10^{-8} M [^{3}H] H_2CB (a concentration close to the K_d of H_2CB for motility-related sites [9]), 50% displacement of the labeled compound was observed at 0.1 μM H_2CB and at 2.2 μM CBAc (Fig. 6). This indicates that CBAc is only 1/20 as effective as H_2CB in competing for motility-related sites.

In order to test whether the higher levels of CBAc required to displace [^{3}H] H_2CB resulted from the lower affinity of the drug for motility-related sites or were due to a reduction in its free concentration caused by its binding to transport-related sites and nonspecific partitioning into the membrane, we performed a competitive binding assay on solublized motility-related sites in a low-salt extract of red cell membranes [9]. This experiment was performed with the isoelectric precipitation assay [10] at 5×10^{-9} M [^{3}H] H_2CB, a concentration approximately equal to the K_d of this drug for motility-related sites measured under the conditions used for this assay (D.C. Lin and S. Lin, unpublished results). We found that 50% displacement of the [^{3}H] H_2CB was observed at 0.02 μM H_2CB and 0.45 μM CBAc, indicating that removing the membrane and transport sites did not cause CBAc to be a more effective competitor in this assay; the derivative is still only about 1/20 as effective as H_2CB.

DISCUSSION

Since CB affects both membrane transport and motile functions of the cell, it has been proposed that the drug acts in a rather nonspecific way by partitioning into membrane lipids

[19] or binding to hydrophobic regions of membrane-associated proteins in general [14]. The data presented here show that modification of the C-7 hydroxyl group of CB has a much greater effect on its action on cell motility than on its ability to inhibit sugar transport. In an earlier study, we have shown that saturation of the C-21-22 double bond of CB abolishes its ability to block sugar transport without significantly affecting its ability to inhibit cell motility [7, 8]. These two groups of experiments demonstrate that the inhibition of sugar transport and cell motility in fibroblasts by CB are independent events, mediated by separate receptors. The CBAc experiments, in particular, argue against the suggestion that inhibition of sugar transport may be mediated by the interaction of CB with cytoskeletal structures[20].

The competitive binding experiments provide an explanation for the effect of acetylation of the C-7 hydroxyl group on the pharmacologic activity of CB. The human red cell was used as the test system in this study because it is possible to measure cytochalasin binding to sugar transport proteins and to cytoskeletal proteins independently. Consistent with its effectiveness in inhibiting sugar transport in red cells and fibroblasts, CBAc was found to displace [^{3}H] CB from transport-related sites with an efficiency about 1/4 of that of CB. In contrast, the derivative was estimated to be only 1/20 as effective as H_2CB in displacing [^{3}H] H_2CB from sites located in cytoskeletal proteins. This finding is in keeping with the observation that CBAc, at a concentration about 10 times higher than the effective level for CB, does not affect fibroblast morphology and motility. Therefore, one can conclude from the data of the competitive binding experiments that the lower potency of CBAc in inhibiting sugar transport and cell motility is a direct result of its lower affinity for CB binding sites which have a high degree of structural specificity for their substrates.

Our present knowledge of the pharmacologic properties of several cytochalasin analogs suggests that the two types of CB effects on fibroblasts can be chemically dissected: the C-7 hydroxyl group is essential for activity against cell motility and the C-20 hydroxyl group is important for activity against sugar transport. As demonstrated in this study, acetylation of the first hydroxyl group produced a derivative (CBAc) which is active only against sugar transport, whereas acetylation of both hydroxyl groups yielded a compound ($CBAc_2$) which is inert against both transport and motile processes. These results complement our earlier observation that H_2CB is effective in inhibiting cell motility but does not inhibit sugar transport [7, 8]. Although this derivative has the C-7 and C-20 hydroxyl groups, the saturation of its double bond at C-21-22 may cause sufficient movement of the oxygen atom at C-20 from its original position that the drug can no longer bind to the sugar transport protein in the manner proposed by Taylor and Gagneja [21].

Our finding that blocking the C-7 hydroxyl group of CB has a relatively minor effect on its activity against sugar transport opens up several possibilities for the preparation of derivatives which could be useful for studying sugar transport in fibroblasts. For instance, the product obtained by linking CB to a solid support (eg, sepharose beads) using a synthetic route similar to that used for preparing CBAc would be useful in affinity chromatography for isolating detergent-solubilized membrane proteins involved in sugar transport. Similarly, the coupling of radioactive, fluorescent, and photoaffinity reagents to the C-7 hydroxyl group could yield compounds helpful for the identification of cytochalasin binding components in experiments in vivo and in vitro.

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