Journal of Supramolecular Structure 11:283–293 (1979)

Specific Interaction of Cytochalasins With Muscle and Platelet Actin Filaments In Vitro

Thomas H. Howard and Shin Lin

Department of Biophysics, The Johns Hopkins University, and Department of Pediatrics, Division of Hematology, The Johns Hopkins Hospital, Baltimore, Maryland 21205

The cytochalasins (CE, CD, CB and H_2CB) inhibit numerous cellular processes which require the interaction of actin with other structural and contractile proteins. In this report we describe the effects of the cytochalasins on the viscosity and morphology of muscle and platelet actin. The cytochalasins decreased the viscosity of F-actin solutions. The effect of H_2CB, CB and CD on F-actin viscosity was maximal at concentrations of 20–50μM and did not increase with time. In contrast, CE caused a progressive decrease in the viscosity of F-actin solutions which was dependent upon the concentration of CE and the duration of incubation of the CE-actin mixture. After two hours of incubation of drug-actin mixtures, the relative effectiveness of the cytochalasins in reducing the viscosity of F-actin was CE>CD>CB=H_2CB. The effects of CD and CE were paralleled by morphologic changes in negatively stained actin filaments. The effects of the cytochalasins on the viscosity and morphology of muscle and platelet actin were the same whether the drugs were added before or after the polymerization of the protein.

These studies show that the interaction of the cytochalasins with actin is highly specific. Because the relative potencies of these drugs for affecting motile processes and the relative affinities of the drugs for binding sites within a variety of cells are CE>CD>CB=H_2CB, the effects of cytochalasins on actin described here may contribute to some of the biological effects of the drugs on motile processes.

Key words: cytochalasins, muscle and platelet actin, microfilaments, cell motility, viscosity changes, electron microscopy

The cytochalasins, a family of alkaloids produced by a variety of fungi, have been found to inhibit a large number of cellular processes which are thought to involve interactions of contractile proteins (for review, see [1]). Studies utilizing electron microscopy and immunofluorescence techniques showed that treatment of cultured mammalian cells with cytochalasin B (CB), by far the most studied congener, results in transformation of actin-containing microfilaments in the cells into amorphous clumps of filamentous materials [eg, 2, 3]. These observations directed the subsequent search for the site of

Received April 25, 1979; accepted July 10, 1979.

0091-7419/79/1103-0283$02.30

action of cytochalasins toward contractile proteins – actin [4], myosin [5], and high molecular weight actin-binding proteins [6, 7].

Several years ago, we presented the first evidence that CB can interact directly with actin molecules; in experiments in vitro, the drug decreased the intrinsic viscosity of muscle F-actin [4] and altered the morphology of platelet F-actin, as shown by electron microscopy [8]. However, because relatively high concentrations of CB (10–100 μM) were used in that study, whether the observed effects have any relevance to the inhibitory effects of the drug on cell motility has remained in question.

More recently our inquiries into the mechanism of action of the cytochalasins have been greatly aided by the synthesis and study of several congeners of CB. We found that in many, but not all, motile systems (eg, axonal growth from neurons, membrane ruffling, cytokinesis, cell spreading, and shape changes in fibroblasts), the relative potencies of the cytochalasins are: cytochalasin E (CE) > cytochalasin D (CD) > dihydrocytochalasin B (H_2CB) ≅ CB [9, 10, and S. Lin, J.C. Scheller, E.L. Onishchuk, M. Grumet and T.H. Howard, manuscript in preparation]. This type of specificity is reflected in the relative affinity of the cytochalasins for high-affinity binding sites located in actin-containing supramolecular complexes purified from human red cell membranes [11, 12] and bovine brain extracts [13]. We were prompted by these findings to examine the effects of CE, CD, H_2CB and CB on the viscosity and the morphology of muscle and non-muscle actin. The results reported here show that, in general, the relative effectiveness of the cytochalasins in altering the structure of purified actin filaments corresponds to the relative potencies of the drugs in affecting cell motility and morphology. This suggests that low-affinity interaction of cytochalasins with actin molecules may contribute to many of the effects of the drugs on cells.

MATERIALS AND METHODS

Cytochalasins

CB, CD, and CE were purchased from Aldrich Chemical Co. H_2CB was prepared by reduction of CB with $NaBH_4$, as previously described [14]. Cytochalasin D diacetate ($CDAc_2$) was prepared by the method of Minato and Matsumoto [15]. On silica gel thin layer chromatography plates $CDAc_2$ migrated as a single spot distinct from CD.

Small volumes of concentrated stock solutions of the cytochalasins dissolved in dimethyl sulfoxide (DMSO) were added to the actin solutions to obtain the desired cytochalasin concentrations. In all of the experiments the final concentrations of the carrier solvent (DMSO) in the actin solutions were below 0.3% (v/v).

Actin Preparations

Muscle actin was isolated from rabbit skeletal muscle by the method of Spudich and Watt [16]. Sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis [17] showed that this preparation was approximately 95% pure. For one experiment, this actin preparation was further purified by chromatography on a Sepharose-4B column equilibrated in 2 mM Tris-HCl,0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, pH 8.0 (at 25°C). The actin-containing fractions from the column were at least 99% free of contaminating proteins, as judged by SDS/polyacrylamide gel electrophoresis.

Platelet actin was isolated from out-dated platelets (generously donated by the Baltimore Regional Red Cross Center) according to the procedure of Gordon et al [18].

SDS/polyacrylamide gel electrophoresis showed that this preparation was at least 99% free of contaminating proteins.

Viscometry

Viscosity of actin solutions was determined with Ostwald-type viscometers with flow time for water of about 30 s. Except where otherwise specified, measurements were made at 25°C. Specific viscosity (η_{sp}) is defined as (η_{rel}-1), where η_{rel} is the flow time for the actin solution divided by the flow time for the corresponding buffer.

In all of the experiments presented in this paper, the muscle actin solutions contained 1 mg protein per ml in 2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, pH 8.0 (at 25°C), with (for F-actin) or without (for G-actin) 50 mM KCl. In the platelet actin experiments, the protein (1 mg/ml) was in 10 mM imidazole, 0.5 mM ATP, 0.1 mM $CaCl_2$, 0.75 mM β-mercaptoethanol, pH 7.5 (at 25°C).

Electron Microscopy

Samples were applied to carbon-coated grids, negatively stained with 1% uranyl acetate, and examined under an electron microscope [8].

RESULTS

Effect of Cytochalasins on Viscosity of Muscle F-Actin

Less than four minutes after the addition of cytochalasins to a solution of muscle F-actin the viscosity of the solution decreases. This immediate effect on F-actin is shown in Figure 1. CB and CD caused a maximum decrease in viscosity at concentrations of

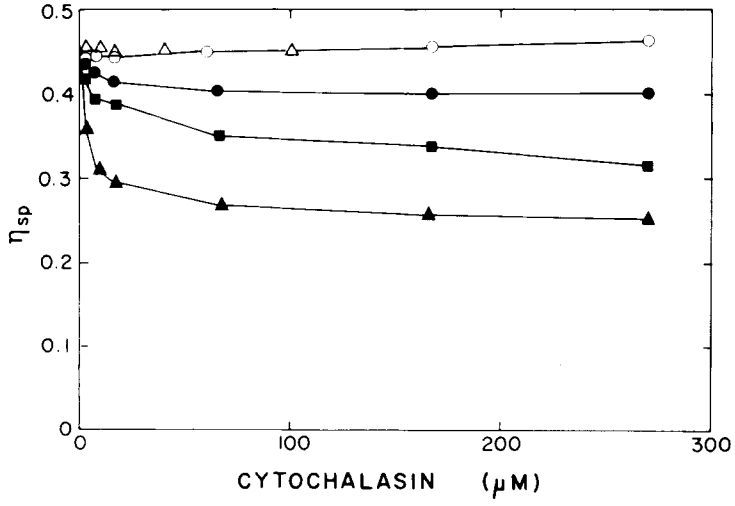

Fig. 1. The immediate effect of cytochalasins on viscosity of F-actin. Increasing concentrations of CB (●), CD (▲), CE (■), $CDAc_2$ (△), or equivalent volumes of the carrier solvent DMSO (○) were added to muscle F-actin. In this experiment increasing amounts of a drug were added to the same actin solution. Within four minutes of the drug addition the viscosity of the solution was determined. Total time for the experiment was 30 min. Similar results were obtained when separate F-actin samples were used for each concentration of drug.

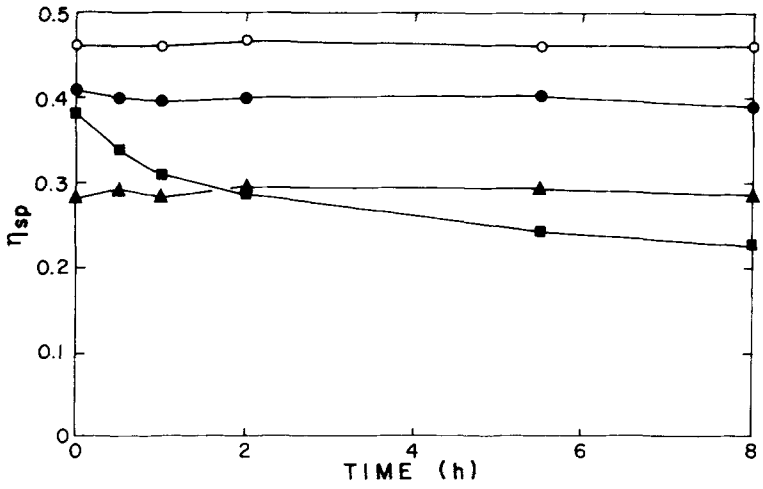

Fig. 2. Time course of the effect of cytochalasins on viscosity of F-actin. At zero time, 20 μM CB (•), CD (▲), CE (■), or an equivalent amount of DMSO (○) was added to a solution of muscle F-actin, and the viscosity of the solution was determined at the indicated times.

20–50 μM. A sharp decrease in viscosity was also produced by CE in the 20–50 μM concentration range, but the viscosity continued to decrease at higher drug levels. H_2CB, which is similar to CB in potency in affecting cell motility and morphology [9, 10], decreased the viscosity in a manner indistinguishable from that of CB (data not shown). In contrast, $CDAc_2$, a biologically inactive derivative which has no effect on 3T3 cell morphology at concentrations up to 100 μM [A. Lees and S. Lin, unpublished data], had no effect on the viscosity of F-actin solutions. In this experiment, the relative potencies of the cytochalasins, as measured by the extent of the decrease in F-actin viscosity immediately after drug addition, are: CD > CE > CB = H_2CB.

The actin preparation used in the preceding experiment contained about 5% contamination from other proteins, as judged by SDS/polyacrylamide gel electrophoresis. When the actin was further purified by Sepharose-4B chromatography, the specific viscosity of a 1 mg/ml solution of F-actin went up from 0.47 to 0.67. The percent decrease in viscosity of this actin preparation in the presence of cytochalasins was comparable to that shown in Figure 1 (data not shown). These results show that further purification of the actin does not affect the ability of the cytochalasins to decrease the viscosity of F-actin.

The time course of the effects of cytochalasins on F-actin viscosity is presented in Figure 2. Maximum decreases in viscosity produced by CB and CD were reached before the first points were taken (< 4 min). Although the onset of the effect of CE on F-actin was equally rapid, the viscosity of the F-actin mixture continued to decrease with time for at least 8 h. At 2 h, the effects of 20 μM CE on actin viscosity exceeded that of 20 μM CD.

As shown in Figure 3, the rate of decrease in F-actin viscosity caused by CE is dependent on drug concentration; F-actin viscosity decreased by 50% in > 12, 9 and 1 h in the presence of 3, 20 and 160 μM CE, respectively. The rate and extent of viscosity decrease were the same whether the CE-actin mixture was incubated at 25°C or 37°C (data not shown). Incubation of CE with buffer for 4 h before mixing with actin did not

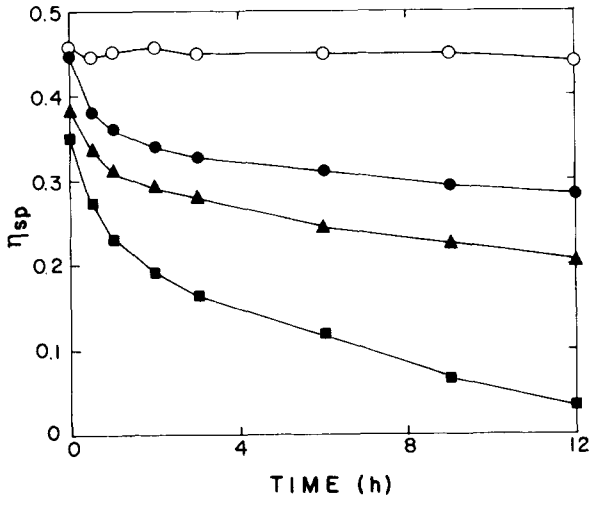

Fig. 3. Relationship of CE concentration and time of incubation to the viscosity of F-actin. At zero time, 3 μM (●), 20 μM (▲), or 160 μM (■), CE, or an equivalent amount of DMSO (○) was added to muscle F-actin, and the viscosity of the solution was determined at the indicated times.

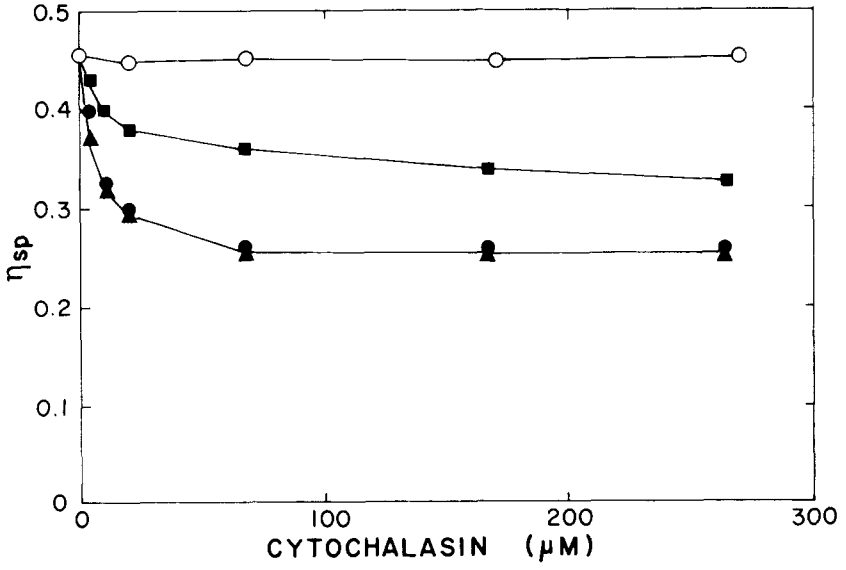

Fig. 4. The effect of simultaneous additions of CD and CE on the viscosity of F-actin. CD (▲), CE (■), a 1:1 mixture of CD and CE (●), or an equivalent amount of DMSO (○) was added to a solution of muscle F-actin. In this experiment, the drug(s) was added in increments to the same actin solution over 30 min, and the viscosity of the resultant solution was determined after each addition.

affect the rate or extent of decrease in viscosity (data not shown), indicating that the potency of CE did not increase with time as a result of chemical reaction.

Since the effect of CE on muscle F-actin is qualitatively and quantitatively different from those of CB and CD, the mode of action of the drugs in this system may be different.

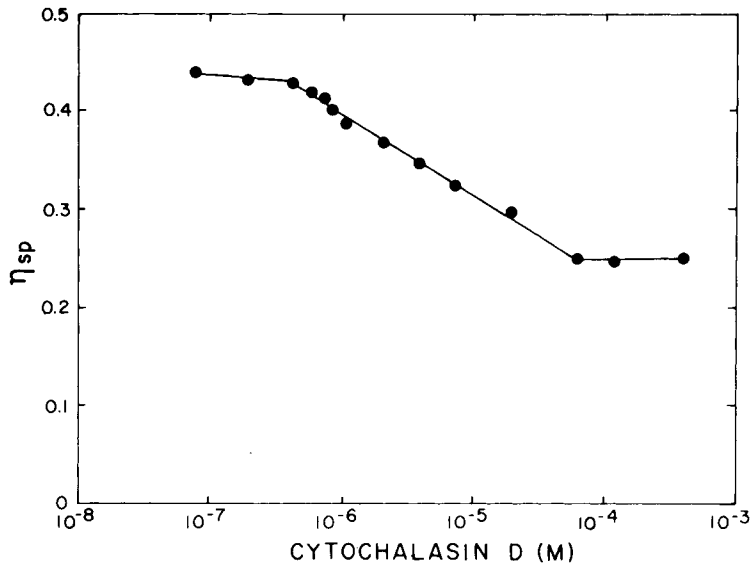

Fig. 5. Effect of concentration of CD on the viscosity of F-actin. The viscosity of a 23 μM muscle F-actin solution (1 mg protein/ml) containing various concentrations of CD was determined.

We tested this possibility by examining the effect of simultaneous additions of CD and CE to F-actin. We found that addition of equimolar concentrations of CD and CE to F-actin had an immediate effect on viscosity equivalent to that of addition of CD alone (Fig. 4); the same result was obtained when CD was added after CE (data not shown). Since the immediate effects of the two cytochalasins were not additive, either the two drugs share a common site of action in this system or the effect of CD on actin filaments pre-empts that of CE.

The dose response of the effect of CD on actin viscosity was accurately determined so that correlation of this effect with the biological action of the drug can be made. The threshold level for the viscosity effect was around 0.5 μM and maximal effect was observed at 50 μM CD (Fig. 5). As noted above $CDAc_2$, a biologically inactive derivative, has no effect on F-actin viscosity. Furthermore, a high concentration (100 μM) of this derivative did not block the effect of CD (3 μM) on actin viscosity, demonstrating that the derivative does not interact with actin molecules (data not shown). These results indicate that the effect of CD on F-actin is specific and saturable. The dose response curve of the action of CB on F-actin viscosity has been published previously [4, 8]. Similar experiments with CE are difficult to perform because of the time dependency of the effect of this drug.

Effect of Cytochalasins on Viscosity of Platelet F-Actin

The effect of cytochalasins on F-actin from a non-muscle tissue was tested by isolating the protein from platelets. Although this actin preparation consisted of two molecular species (β and γ) which are distinct from muscle actin (α) in isoelectric point [18] the effects of CB, H_2CB, CD and CE on its viscosity were essentially the same as those observed with muscle F-actin (data not shown).

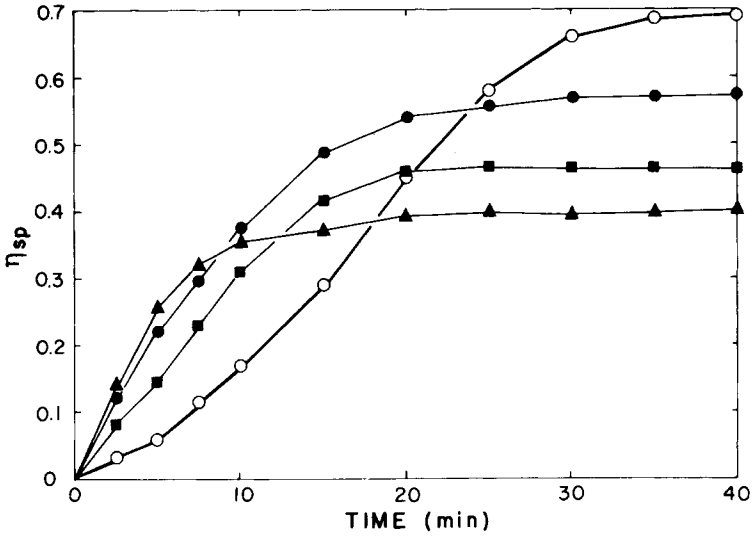

Fig. 6. Effect of cytochalasins on viscosity changes accompanying polymerization of muscle actin. At zero time, muscle G-actin was induced to polymerize by addition of 50 mM KCl in the presence of 20 μM CB (●), CD (▲), CE (■), or an equivalent amount of DMSO (○). Viscosity of the resultant solution was determined at the indicated times.

Effect of Cytochalasins on Actin Viscosity During Polymerization

Studies on the effect of cytochalasins on polymerization of G-actin induced by KCl confirmed and extended the results presented in the preceding sections. In addition to decreasing the final viscosity of F-actin, the cytochalasins caused the viscosity changes associated with the polymerization of actin to occur more rapidly than in the control. As shown in Figure 6, a rapid change in viscosity occurred in the first 10–15 min after polymerization of the G-actin had been induced by addition of 50 mM KCl. The rate of the initial increase in viscosity was approximately 0.05/min in the presence of CB or CD, 0.03/min in the presence of CE, and 0.01/min in the absence of the drugs. Consistent with the time dependence of the effect of CE on F-actin viscosity (Figs. 2 and 3), the effect of this congener on the final viscosity of the CE-actin mixture was dependent on drug concentration (data not shown) and duration of incubation of the drug with G-actin prior to induction of polymerization. As shown in Figure 7, both the initial rate of increase in viscosity and the final level of viscosity of the CE-actin mixture decreased with increasing time of incubation. With 4 hours of pre-incubation, the initial rate of increase in viscosity fell below that of the control sample.

Effect of Cytochalasins on Morphology of Actin Filaments

Platelet F-actin treated with cytochalasins was negatively stained with 1% uranyl acetate and examined under an electron microscope. Distinctive morphological changes in actin filaments induced by the drugs were noted. In general, the severity of the morphological alterations of the filaments correlated with the degree of reduction in F-actin viscosity. Whereas CB at 50 μM caused minimal clumping and some twisting of individual filaments, those filaments treated with 50 μM CD or CE were markedly

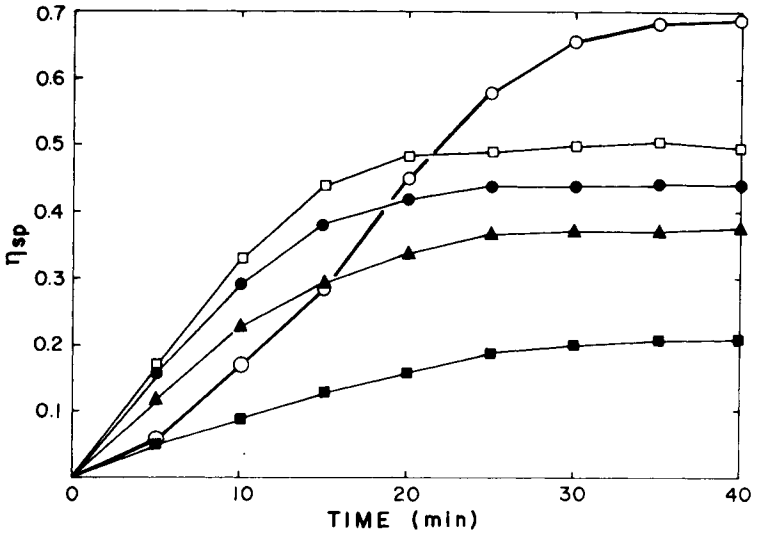

Fig. 7. Effect of preincubation on the effect of CE on viscosity changes accompanying muscle actin polymerization. Muscle G-actin (2mg/ml) was preincubated in 40 μM CE for 0 h (□), 0.25 h (●), 1 h (▲), 4 h (■) or DMSO (○) for 4 h. At zero time, actin polymerization was induced by addition of an equal volume of buffer containing 100 mM KCl, and the viscosity of the solution was determined at the indicated times.

clumped and individual filaments were gnarled and twisted (See Fig. 8). Thus, at a given concentration, the effect of CD and CE exceeded that of CB. Finally, CE-treated actin with a viscosity 35% of that of control (obtained by incubation with the drug for 6 h), showed only occasional presence of filaments and numerous amorphous globs of negatively stained material. The above observations were also obtained when muscle actin was used and when the cytochalasins were added before or after induction of actin polymerization.

DISCUSSION

The availability of several cytochalasins and their derivatives has added a new dimension to the identification of cellular target sites of CB. We have previously determined that the relative potencies of the cytochalasins in affecting many, but not all, forms of motile processes follow a certain order: CE > CD > CB = H_2CB [9, 10; S. Lin, J.C. Scheller, E.C. Onishchuk, M. Grumet, and T.H. Howard, manuscript in preparation]. Therefore, excepting drastic differences in the rate of membrane permeation and catabolism of the different congeners [for discussion see reference 19], interaction of these drugs with their target sites in vitro should reflect the same order of potencies.

The studies presented here support the contention that interaction of cytochalasins with actin molecules contributes to some of the biological effects of the drugs on cells. First, we showed by viscometry and electron microscopy that the effect of the cytochalasins on F-actin structure in vitro is a highly specific one. In general, the relative potency of a drug in these experiments correlates well with its relative potency in biological systems. The only exception is the observation that the immediate effect

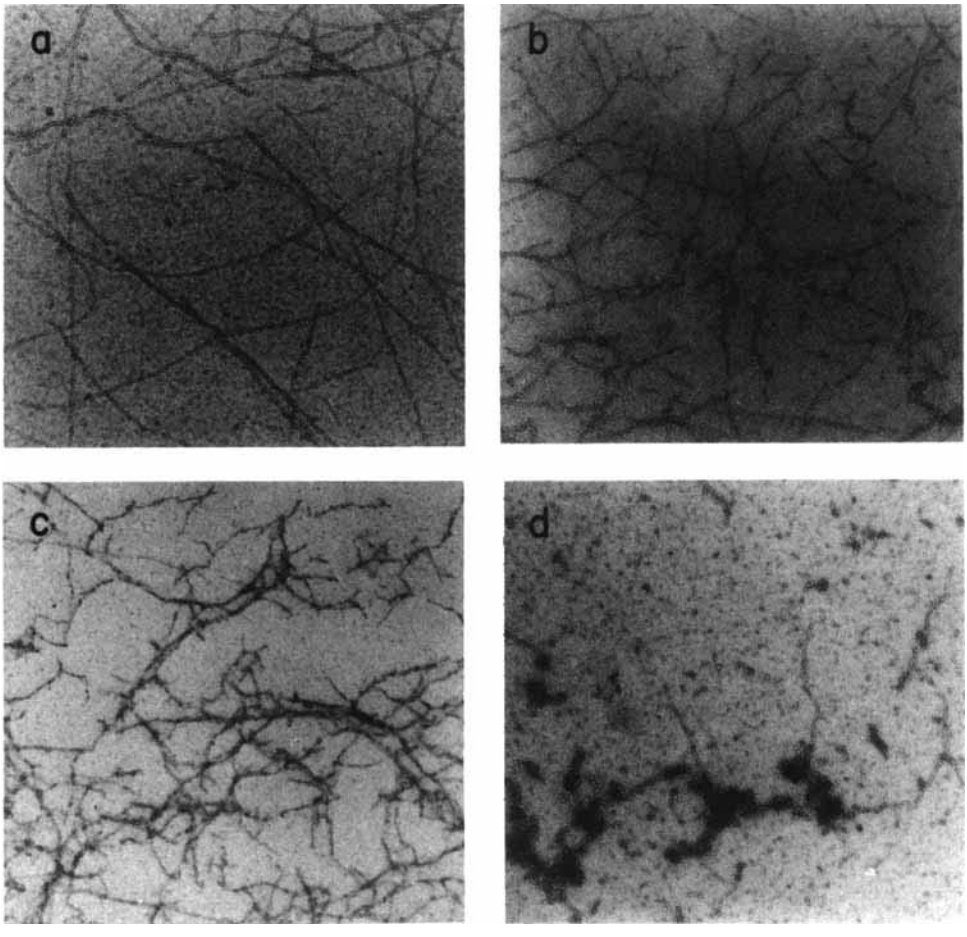

Fig. 8. Negatively stained platelet actin filaments immediately (< 5 min) after addition of (a) DMSO, (b) 50 μM CD, (c) 50 μM CE and six hours after addition of 50 μM CE (d) (magnification, × 30,000).

of CE on F-actin is less than that of CD. However, in time, the effect of CE does exceed that of CD. Although we have no explanation for the time dependency of the CE effect, we have shown that this phenomenon is not caused by the chemical conversion of the drug to a more active form. Second, the low concentration of CD (0.5–50 μM) required to decrease F-actin viscosity is in the range that affects cell motility and morphology [9, 10, 20]. Third, consistent with earlier results obtained with CB [8], electron microscopy showed that CD and CE also affect the morphology of actin filaments in a manner resembling the disruption of actin-containing microfilaments in cells exposed to these drugs [eg, 3, 20, 21].

The viscosity effect described in this paper is likely to be a reflection of direct interaction of cytochalasins with actin molecules. This contention is supported by the observations that maximal decreases in viscosity occur at near stoichiometric levels of the drugs, and that removal of minor protein contaminants had no effect on the viscosity changes. Low and Dancker [22] have also studied the effect of CB on the viscosity of

muscle F-actin. They found that actin polymerized overnight at 4°C in the presence of Mg^{++} and ADP exhibited decreased viscosity when treated with 200 μM CB. This effect was abolished by the presence of 90 mM KCl. Pairing these observations with data on ATP hydrolysis [23] they hypothesized that CB has its maximal effect on the viscosity of incompletely polymerized actin. In contrast, we showed here that the effect of CB on F-actin viscosity in 50 mM KCl is comparable to the effect observed with 100 mM KCl [4]. The differences in our observations and those of Low and Dancker may be due to the different conditions utilized for actin polymerization. Reconciliation of these observations awaits a more exact definition of the incompletely polymerized state of actin – a transitional state through which actin must pass as it is polymerized and depolymerized in living cells.

The electron micrographs of cytochalasin-treated actin filaments indicate that the drugs enhance association among actin filaments, making them aggregate or coil up into structures which have an axial ratio lower than that of fully extended filaments. The higher rate of increase in viscosity associated with actin polymerization in the presence of CB was first noted by Low and Dancker [23]. As shown in this report a similar effect is seen with CE and CD. This phenomenon may represent a true stimulation of actin polymeri zation by cytochalasins or an aggregation of newly formed, short filaments into elongated, multifilament structures with axial ratios higher than those of the individual short filaments.

It has been previously demonstrated that CB has no effect on the viscosity of actin filaments which also contain tropomyosin-troponin [8]. This may explain why muscle myofibrils and fibroblast microfilament bundles containing both actin and tropomyosin [24] are insensitive to CB [25, 26]. Recent experiments performed in our laboratory have shown that there is a class of high-affinity CB binding sites located in actin-containing supramolecular complexes purified from human red cell [12] and bovine brain [13]. Relative affinities of cytochalasins for these sites correspond to the relative potencies of the drugs in affecting cell motility and morphology (ie, CE > CD > CB = H_2CB [10]). Although actin molecules are an integral part of those complexes, G-actin contains only low affinity binding sites for CB [27]. Thus, it appears that CB can affect cell motility and morphology by interacting with one or both types of cellular target sites: the high-affinity sites located in actin-containing complexes and the low-affinity sites located in actin molecules.

ACKNOWLEDGMENTS

This work was supported by research grant GM-22289, research career development award GM-00241 (to SL), and a biomedical sciences support grant from the National Institutes of Health. T.H.H. was supported by NIH hematology training grant HL-07143.

REFERENCES

1. Tannenbaum SW (ed): "Cytochalasins: Biochemical and Biological Aspects." Amsterdam: North Holland, 1978.
2. Weber K, Ratke P, Osborn M, Franke W: Exp Cell Res 102:285, 1976.
3. Wessells N, Spooner B, Ash J, Ludena M, Wrenn J, Taylor E, Bradley O, Yamada K: Science 171:135, 1971.
4. Spudich J, Lin S: Proc Natl Acad Sci USA 69:442, 1972.
5. Puzkin E, Puzkin S, Lo L, Tannenbaum S: J Biol Chem 248:7754, 1973.

6. Hartwig J, Stossel T: J Cell Biol 71:295, 1976.
7. Weihing R: J Cell Biol 71:303, 1976.
8. Spudich J: Cold Spring Harbor Symposia on Quantitative Biology XXXVII:585, 1972.
9. Atlas SJ, Lin S: J Cell Biol 76:360, 1978.
10. Lin S, Lin D, Flanagan M: J Biol Chem 253:1415, 1978.
11. Lin S, Lin D: J Biol Chem 253:1415, 1978.
12. Lin DC, Lin S: Proc Natl Acad Sci USA 76:2345, 1979.
13. Flanagan MD: Fed Proc 38:3392, 1979.
14. Lin S, Snyder C: J Biol Chem 252:5464, 1977.
15. Minato H, Matsumoto N: J Chem Soc (c):38, 1970.
16. Spudich J, Watt S: J Biol Chem 246:4866, 1971.
17. Weber K, Osborn M: J Biol Chem 244:4406, 1969.
18. Gordon D, Boyer J, Korn E: J Biol Chem 252:8300, 1977.
19. Lin S: In Tannenbaum SW (ed): "Cytochalasins: Biochemical and Biological Aspects." Amsterdam: North Holland, 1978, p 499.
20. Miranda A, Godman G, Tannenbaum S: J Cell Biol 62:406, 1974.
21. Schroeder TE: J Cell Biol 53:419, 1972.
22. Low I, Dancker P, Wieland Th: FEBS Lett 54:263, 1975.
23. Low I, Dancker P: Biochim Biophys Acta 430:366, 1976.
24. Lazarides E: J Cell Biol 68:202, 1976.
25. Sanger J, Holtzer S, Holtzer H: Nature New Biol 229:121, 1971.
26. Goldman R: J Cell Biol 52:246, 1972.
27. Lin S, Spudich J: J Supramol Struct 2:228, 1970.