

# Correlation between Effects of 24 Different Cytochalasins on Cellular Structures and Cellular Events and Those on Actin In Vitro

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**ABSTRACT** To compare the effects of cytochalasins on the cellular level with those on the molecular level, 24 cytochalasins, 20 natural compounds and 4 derivatives, were used. The following effects were tested for each of 24 cytochalasins: (a) four high dose (2–20  $\mu$ M) effects on the cellular level: rounding up of fibroblastic cells, contraction of actin cables, formation of hairy filaments containing actin, and inhibition of lymphocyte capping; (b) a low dose (0.2–2  $\mu$ M) effect: inhibition of membrane ruffling; and (c) two in vitro effects: an inhibition of actin filament elongation (the high affinity effect [low dose effect] in vitro) and an effect on viscosity of actin filaments (the low affinity effect [high dose effect] in vitro). These results indicated that there are almost the same hierarchic orders of relative effectiveness of different cytochalasins between low and high dose effects and between cellular and molecular effects. From the data obtained with the 24 cytochalasins, we have calculated correlation coefficients of 0.87 and 0.79 between an effect in vivo, inhibition of capping, and an effect in vitro, inhibition of actin filament elongation, as well as between inhibition of capping and another effect in vitro, effect on viscosity of actin filaments, respectively. Furthermore, a correlation coefficient between the high affinity effect and the low affinity effect determined in vitro was calculated to be 0.90 from the data obtained in this study. The strong positive correlation among low and high dose effects in vivo and those in vitro suggests that most of the effects caused by a cytochalasin, irrespective of doses or affected phenomena, might be attributed to the interaction between the drug and the common target protein, actin.

In the course of the immunofluorescence microscope study on cytochalasin-treated cells using actin antibody, we have found that aspochalasin D, a 10-isopropylcytochalasin, strongly induced the formation of rodlets containing actin in the cytoplasm of the treated fibroblasts. In contrast, other cytochalasins, including cytochalasin B, cytochalasin C, cytochalasin D, and cytochalasin H, were found to induce the formation of nuclear rodlets. Among them, cytochalasin D was the most potent in the ability to induce the rodlets. Both cytoplasmic and nuclear rodlets found in the cytochalasin-treated cells were similar in ultrastructures to those induced by 5 to 10% (vol/vol) dimethyl sulfoxide in the same type of cells.

The secondary metabolites of molds known as cytochalasins inhibit a variety of cellular movements, including cell division, motility, secretion, and phagocytosis, and cause change in cell shape (45). In addition, some, but not all, cytochalasins inhibit sugar transport by competing with sugars for binding to high-affinity sites in the plasma membrane (5, 34). The inhibition of

sugar transport by the drugs is not related to the effect on cellular structures containing actin and other contractile proteins (5, 26). Apart from the inhibition of sugar transport, it appears there might be two types of cytochalasin B (CB) effects on cultured cells which are distinguishable according to the effective dose of the drug or to the effective length of an

exposure time to the drug. Brief treatment or treatment with low dose ( $<1 \mu\text{M}$ ) of CB resulted in an instantaneous inhibition of membrane ruffling and cell migration but not a change in gross cell morphology (16). The same phenomena have been described in ultrastructural terms, showing that CB disrupted the lattice or network structures of microfilaments but not the sheath structures of the same filaments (42). However, treatment with 10–20  $\mu\text{M}$  CB of spreading cells on substratum induced a change in cell morphology from flat to arborized cells (37). An analysis of this relatively high dose effect of CB by the immunofluorescence method using actin antibody has shown that actin cables, which correspond to the sheath described above, were dissociated, and most of the actin formed dense aggregates or asterisklike patches (46).

Recent evidence suggests that the inhibition of cellular movements, a low dose effect of CB, may be attributed to the binding of cytochalasins to high-affinity sites located in a high molecular weight complex consisting mainly of actin (26). More recently, several groups have found that substoichiometric doses of CB and other cytochalasins blocked the polymerization induced by salts of skeletal muscle actin (8, 9, 13, 27, 29). Viscometric observations suggest and electron microscope analysis (29) confirm that the CB inhibits elongation of actin filaments, probably by interacting with the polymerizing end of the filament (8, 9, 13, 29). Thus, molecular interactions in vitro between cytochalasins, particularly CB and actin, have been clarified to some extents. However, whether or not cellular effects of cytochalasins are accounted for by the same interactions remains to be elucidated.

It has been suggested that if the molecular mechanism for an in vitro action of cytochalasins is the same as that which works in an in vivo effect of the same cytochalasins, the relative effectivenesses of different cytochalasins in vivo must reflect those seen in the in vitro action (9, 19, 26, 29). So far, three or four cytochalasins, including CB, cytochalasins D and E (CD and CE), and dihydro-CB, have been used for comparisons between their effects on molecular and cellular levels. In this study, we used 24 cytochalasins, 20 natural compounds and 4 derivatives, and examined their effects on cellular structures containing actin, cellular events which involve actin, and actin polymerization in vitro. We found, with only a few exceptions, almost the same hierarchic orders of effectiveness of different cytochalasins at low and high doses in vivo as we found in vitro. We discuss the results in terms of the mechanism for the in vivo effects of cytochalasins.

## MATERIALS AND METHODS

### Cytochalasins

The names of cytochalasins are generally used in the abbreviated forms shown in Table I. The basic skeletal structure of cytochalasins is shown in Fig. 1 (31, 45). (a) CA, CC, CE, and CG (2, 3, 4, 10), (b) CB and deoxaphomin (7, 36), (c) CD (197), (d) CH and CJ (6, 33), (e) chaetoglobosin K (ChK) (43), and (f) aspochalasin B and D (AsB and AsD) (22) were provided by D. C. Aldridge (Imperial Chemical Industries, Ltd., Macclesfield, England), Ch. Tamm (Institut für Organische Chemie der Universität Basel, Basel, Switzerland), H. Minato (Shionogi Co., Osaka, Japan), R. J. Cole (National Peanuts Research Laboratories, Dawson, Ga.), H. G. Cutler (Agriculture Researches, U. S. Department of Agriculture, Tifton, Ga.), and W. Keller-Schierlein (Eidgenössischen Technischen Hochschule, Zurich, Switzerland), respectively. Chaetoglobosins except ChK were isolated from *Chaetomium globosum* and chemically modified, if necessary, as described previously (31, 39–41).

Cytochalasins were first dissolved in dimethyl sulfoxide (DMSO) at 2 mM and diluted with the medium to give the concentration indicated. The solvent at 1% (vol/vol) or lower concentrations did not affect experimental systems used in

our study at all. Ethanol was also used as a solvent for a few cases in which the solvent effect of DMSO was critically examined.

### Antibodies

Fluorescein isothiocyanate conjugated goat anti-rabbit IgG (FITC-G-anti-Rlg) and FITC-conjugated rabbit anti-mouse IgG (FITC-R-anti-Mlg) were products of Miles-Yeda Ltd. (Rehovot, Israel). Rabbit antibodies directed against chick embryonic fibroblasts actin (actin antibody) and porcine brain tubulin (tubulin antibody) were described previously (50, 51). Antibodies directed against chick embryonic fibroblast myosin and chick gizzard filamin were produced in rabbits, respectively (unpublished results). Rabbit anti- $\alpha$ -actinin raised against porcine skeletal muscle  $\alpha$ -actinin (24) and rabbit anti-tropomyosin (23) were provided by E. Lazarides (California Institute of Technology, Pasadena, Calif.).

### Cells

C3H cells, a mouse fibroblastic cell line (52), were cultured in DMEM–10% FCS containing 100  $\mu\text{g}/\text{ml}$  Kanamycin sulfate (Meiji Seika Kaisha Ltd., Tokyo, Japan). Preparation of lymphocyte suspension in phosphate-buffered saline (PBS) from mouse spleens were described elsewhere (48).

### Cell Morphology

**ROUNDING UP:** Trypsinized C3H cells were plated in 35-mm plastic culture dish ( $4 \times 10^4$  cells/dish) containing DMEM–10% FCS and incubated at  $37^\circ\text{C}$  for 4 h. Cytochalasins were added when indicated and the effects of the drugs on cell morphology were determined 30 min later.

**MEMBRANE RUFFLING:** Membrane ruffles are cell structures related to cell locomotion, which are sharp thickenings of the lamella projecting in the medium, and appear in plan view by phase-contrast microscopy as dark sinuous lines usually at the leading edge (1). C3H cell cultures were prepared as described above, and were used 6 h after the plating. 10 min after the addition of cytochalasins, we examined whether or not membrane rufflings were affected, using a phase-contrast microscope equipped with a  $\times 40$  objective lens.

### Immunofluorescence Observations of Cellular Actin

The distribution of actin in untreated and cytochalasin-treated fibroblasts was examined with actin antibody by the indirect immunofluorescence methods (25). Cells grown on cover slips were treated with cytochalasins, and fixed in 3.7% formaldehyde in PBS at room temperature for 20 min. They were then washed with PBS, treated with 0.2% Triton X-100 in PBS, and labeled with actin antibody as described before (51). Conditions for fluorescence microscopy observations were described elsewhere (50).

### Decoration with Heavy Meromyosin (HMM)

Rabbit skeletal muscle HMM was prepared as described (47). Cytochalasin-treated cells were glycerinated and labeled with HMM (5 mg/ml) according to the method of Ishikawa et al. (20).

### Electron Microscopy

C3H cells attached to plastic dishes were fixed *in situ* in the Karnovsky's paraformaldehyde–glutaraldehyde fixative (21) for 20 min and postfixed with 2%  $\text{OsO}_4$  in 0.1 M collidine buffer (pH 7.2) for 30 min at room temperature. The fixed cells were washed in distilled water, stained with 4% uranyl acetate in water for 4 h, and dehydrated in ethanol. The cells were then treated with propylene oxide to detach from the dishes. Floating cells were collected by centrifugation, washed again in propylene oxide, and embedded in Epon 812 mixtures. Ultrathin sections were made using a Porter-Blum MT-2 ultratome with a diamond knife, and stained with lead acetate. The specimens were examined in a JOEL JEM-100C or JEM-200B electron microscope.

### Capping

Mouse splenic lymphocytes ( $2 \times 10^7/\text{ml}$ ) were used for capping experiments in PBS with or without cytochalasins. An aliquot (0.2 ml) of cell suspension, which was preincubated with or without cytochalasins at room temperature for 10 min unless otherwise indicated, was incubated with 100  $\mu\text{g}/\text{ml}$  FITC-R-anti-Mlg at  $37^\circ\text{C}$  for 10 min. The cells were spun down, and resuspended in the same medium containing 20 mM sodium azide. Determination of Ig-positive cells and those showing capping was described previously (48).

TABLE I  
Effect of Cytochalasins

Drugs	(1) Cell rounding up	(2) Inhibition of membrane ruffling	(3) Actin-cables contraction	(4) Formation of hairy structures	(5) Inhibition of capturing %	(6) Inhibition of actin filament elongation %	(7) Decrease in viscosity of actin filaments %
<b>10-phenylcytochalasins</b>							
Cytochalasin A (CA)	++	++	+	±	95	63	22
Cytochalasin B (CB)	+	+	+	+	47	44	9
Cytochalasin C (CC)	++	+	+	+	64	63	27
Cytochalasin D (CD)	++	++	+	+	79	88	62
Cytochalasin E (CE)	++	++	+	-	94	77	52
Cytochalasin H (CH)	++	+	+	+	73	69	25
Cytochalasin J (CJ)	+	+	+	+	41	42	23
Deoxaphomin	+	+	+	+	70	66	12
<b>10-indolylcytochalasins</b>							
Cytochalasin G (CG)	+	+	+	+	27	32	5
Chaetoglobosin A (ChA)	++	++	+	-	98	85	43
Chaetoglobosin B (ChB)	++	++	+	±	93	80	36
Chaetoglobosin C (ChC)	+	+	+	+	59	28	24
Chaetoglobosin D (ChD)	++	++	+	-	93	84	49
Chaetoglobosin E (ChE)	+	+	+	+	49	33	5
Chaetoglobosin F (ChF)	+	+	+	+	67	48	6
Chaetoglobosin J (ChJ)	++	++	+	-	90	77	38
Chaetoglobosin K (ChK)	-	++	-	-	93	71	31
ChA monoacetate (ChA-Ac)	++	++	+	-	97	79	40
ChD diacetate (ChD-Ac)	+	+	+	-	60	34	10
ChE diacetate (ChE-Ac)	-	-	-	-	20	7	0
ChF monoacetate (ChF-Ac)	±	+	±	±	24	20	3
ChJ monoacetate (ChJ-Ac)	++	++	+	-	79	45	23
<b>10-isopropylcytochalasins</b>							
Aspochalasin B (AsB)	+	+	+	±	39	8	0
Aspochalasin D (AsD)	+	+	+	+	60	13	0

### Preparation of Actin

Rabbit skeletal muscle actin was purified in buffer A (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.5 mM  $\beta$ -mercaptoethanol, 0.2 mM  $\text{CaCl}_2$ ) according to the method described by Spudich and Watt (44). The polymerization-depolymerization method was followed by gel filtration with Sephadex G-100. This actin preparation showed a single peak corresponding to the molecular weight of 42,000 in SDS PAGE. The stock solution of actin was centrifuged at 100,000 g for 60 min before use to remove precipitable fractions. The concentration of actin was determined using bovine serum albumin solution made up in buffer A as standard by the method of Lowry et al. (28) after dilution with water.

### Actin Filament Elongation

Purified actin (1 mg/ml) was fully polymerized at 25°C in buffer A containing 2 mM  $\text{MgCl}_2$ . 2 ml of actin monomer solution (1 mg/ml) in buffer A were mixed with 50  $\mu$ l of fully polymerized actin solution and 20  $\mu$ l of 0.05 M  $\text{MgCl}_2$  solution. Cytochalasins, which had been dissolved in DMSO at 0.2 mM, were added at 2  $\mu$ M to the actin monomer solution just before the addition of polymerized actin and  $\text{MgCl}_2$ . DMSO in the concentration of 1% (vol/vol) did not affect the rate of actin polymerization. Viscosity of the solution was monitored up to 40 min. An average increase in specific viscosity per minute determined between 10 and 20 min after the start of the polymerization was used in this study as a parameter which indicates the rate of actin filament elongation.

### Addition of Cytochalasins to Polymerized Actin Solution

To 2 ml of fully polymerized actin solution (1 mg/ml) in buffer A containing 2 mM  $\text{MgCl}_2$  we added 20  $\mu$ l of cytochalasin solution made up at 2 mM in DMSO. Viscosity of the mixture was monitored up to 40 min. DMSO alone was tested but was found not to decrease the specific viscosity of the actin solution.

### Viscometry

Viscosity was measured at 25.0°C using an Ostwald-type viscometer (Towa Kagako Co., Tokyo, Japan) with buffer flow time of 24 s. The specific viscosity is defined as  $t/t_0 - 1$ , where  $t$  is the flow time of the actin solution and  $t_0$  is the flow time of the buffer.

## RESULTS

### Alteration in Cell Morphology

Cell morphology of C3H cells treated with different doses (0.2 to 20  $\mu$ M) of cytochalasins for 0.5 to 2 h was examined. We used “++,” “+,” or “-” for indicating the extent to which each drug affected the cell morphology (Table I, column 1). When a drug at 2  $\mu$ M hardly induced rounding up of C3H cells within 1 h, but the same drug at 20  $\mu$ M caused rounding up, the effect of the drug was expressed as “+.” When a drug was effective as low as 2  $\mu$ M, the effect of the drug was expressed as “++.” When rounding up of cell body was not induced with a drug at any doses up to 20  $\mu$ M within 1 h, the effect was expressed as “-.” For example, ChE (see abbreviations in Table I) at 2  $\mu$ M did not induce rounding up of cells within 1 h, whereas the same drug at 20  $\mu$ M caused morphological changes and induced 30 to 40% of the treated cell to round up within the same period of incubation. In contrast, ChJ at 2  $\mu$ M induced rounding up of >60% of the cells within 0.5 h. The effect of ChE and ChJ on cell morphology was expressed as “+” and “++,” respectively.

As has been reported (16), the effect of CB or CD is

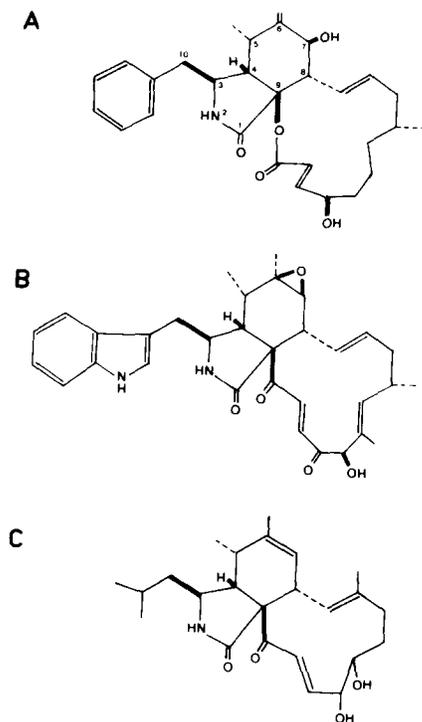


FIGURE 1 Molecular structures of three classes of cytochalasins. (A) A 10-phenylcytochalasin (Cytochalasin B). (B) A 10-indolylcytochalasin (ChA). (C) a 10-isopropylcytochalasin (AsD).

reversible. However, among the drugs tested in this study, ChA, ChB, ChD, ChJ, ChA-Ac, and ChD-Ac caused irreversibly rounding up of cells. When treated with 2  $\mu\text{M}$  or higher concentrations of these drugs, rounded cells did not spread again over substratum after the removal of the drugs. Although the viability of these rounded cells maintained over 90% up to 24 h as long as the cells were not detached from substratum, they became gradually to float in the medium and died over a period of 24–48 h.

### Inhibition of Membrane Ruffling

An inhibition of cell locomotion by micromolar CB is associated with a cessation of membrane ruffling caused by the same drug (16). We have tested the ability of each cytochalasin to inhibit membrane ruffling exhibited by C3H cells. Cytochalasins were added at 0.2 and 2  $\mu\text{M}$  to cultures of C3H cells 6 h after trypsinization and plating. Cells showing membrane ruffling were observed with a phase-contrast microscope before and after the addition of the drugs. The result was expressed as: (a) “++,” when ruffles disappeared by incubating for 10 min with a cytochalasin at both 0.2 and 2  $\mu\text{M}$ ; (b) “+,” when ruffles disappeared by incubating for 10 min with a drug at 2  $\mu\text{M}$ , but not at 0.2  $\mu\text{M}$ ; and (c) “–,” when any detectable effect on membrane ruffling was not observed by 0.2 or 2  $\mu\text{M}$  cytochalasin.

### Alteration in Actin Distribution Revealed by Immunofluorescence Microscopy

**CONTRACTION, ARBORIZATION, AND FORMATION OF HAIRY STRUCTURES:** Fibroblastic cells grown on a substrate contain well-organized actin cables (also referred to as stress fibers or microfilament bundles), which can be visualized by immunofluorescence microscopy using actin antibody (25). For in-

stance, a C3H fibroblastic cell shown in Fig. 2 shows a parallel array of actin cables and a speckled distribution of actin around the nucleus. When C3H cells were treated with 1  $\mu\text{M}$  CB for 30 min, no remarkable change in gross morphology of cell was detected.

As described in previous reports by others (37, 46), when treated with 10–20  $\mu\text{M}$  CB for 30 min, cells contracted and arborized (Fig. 3). Actin cables were still seen on a considerable population (50–80%) of the CB-treated cells, whereas hairy (32) or asterisklike (46) structures were observed on fully arborized cells (Fig. 3).

The contraction of actin cables and cell body was more significantly observed on cells treated for a short period with more potent cytochalasins, especially some 10-indolylcytochalasins. When treated either with 1–20  $\mu\text{M}$  ChA, ChB, ChD, or ChJ, a majority of the cells contracted strongly within 10 min (Fig. 4). The contraction appeared to occur along the stretch of actin cables, and toward the center of each cell, resulting in rounding up of cell body. In some cells, the contraction caused cells to tear apart, leaving their peripheral parts firmly attached to the substratum (Fig. 4) or causing internal tearing (data not shown).

Some cytochalasins at 20  $\mu\text{M}$  including CB, CC, CD, CH, CJ, CG, ChB, ChF, and AsD did, but CE, ChA, ChB, ChD, or ChJ at 20  $\mu\text{M}$  did not induce the formation of the hairy structures (Table I, column 4).

When cells were treated with 20  $\mu\text{M}$  ChK for 30 min to 1 h, neither the contraction nor the formation of hairy structures were induced. It was found, however, that, in contrast to a straight array of actin cables seen in untreated cells (Fig. 2), actin cables were curved in ChK-treated cells (Fig. 5).

In summary, contraction of actin cables followed by cell rounding up, appeared to be common for all the cytochalasins tested (Figs. 3, 4, and 6–9), except ChK, irrespective of the potency of the drugs. Cytochalasins which revealed strong cellular effects in Table I, column 1 and 2, did not or did to lesser extents, if any, induce the formation of hairy structures. An exception for this rule is CD.

**RODLET FORMATION INDUCED BY CYTOCHALASINS:** Among 24 cytochalasins tested in this study, AsD, an 10-isopropylcytochalasin was found to reveal a peculiar effect on cellular structures containing actin. When C3H cells were treated with 20  $\mu\text{M}$  AsD for 30 min, a number of rodlets 0.1–1  $\mu\text{m}$  wide and 0.5–20  $\mu\text{m}$  long containing actin were detected in the cytoplasm of treated cells (Fig. 6). Similar cytoplasmic rodlets containing actin have been previously observed in CA-treated mouse 3T3 cells (35). The rodlets were not stained with antibodies directed against myosin, filamin,  $\alpha$ -actinin, tropomyosin, or tubulin (data not shown). The frequency of cells containing rodlets varied depending upon the concentration of the drug, the length of incubation and the cells used in the experiment. Although the detailed results will be published elsewhere (I. Yahara, F. Harada, and W. Keller-Schierlein, manuscript in preparation), the results indicated that AsD was effective as low as 2  $\mu\text{M}$  when incubated for 30 min. At the optimum conditions where cells were incubated with 10–20  $\mu\text{M}$  AsD for 1 h, rodlets were found in >70% of the treated cells. Number of rodlets within a single cell was 1–127.

A fine structure of the rodlets was investigated by electron microscopy. The results indicated that the rodlets revealed by immunofluorescence microscopy with actin antibody were bundles of microfilaments 5–8 nm in diameter (Fig. 10). Since the rodlets were found by immunofluorescence microscopy to disappear on AsD-treated and then glycerinated cells, the

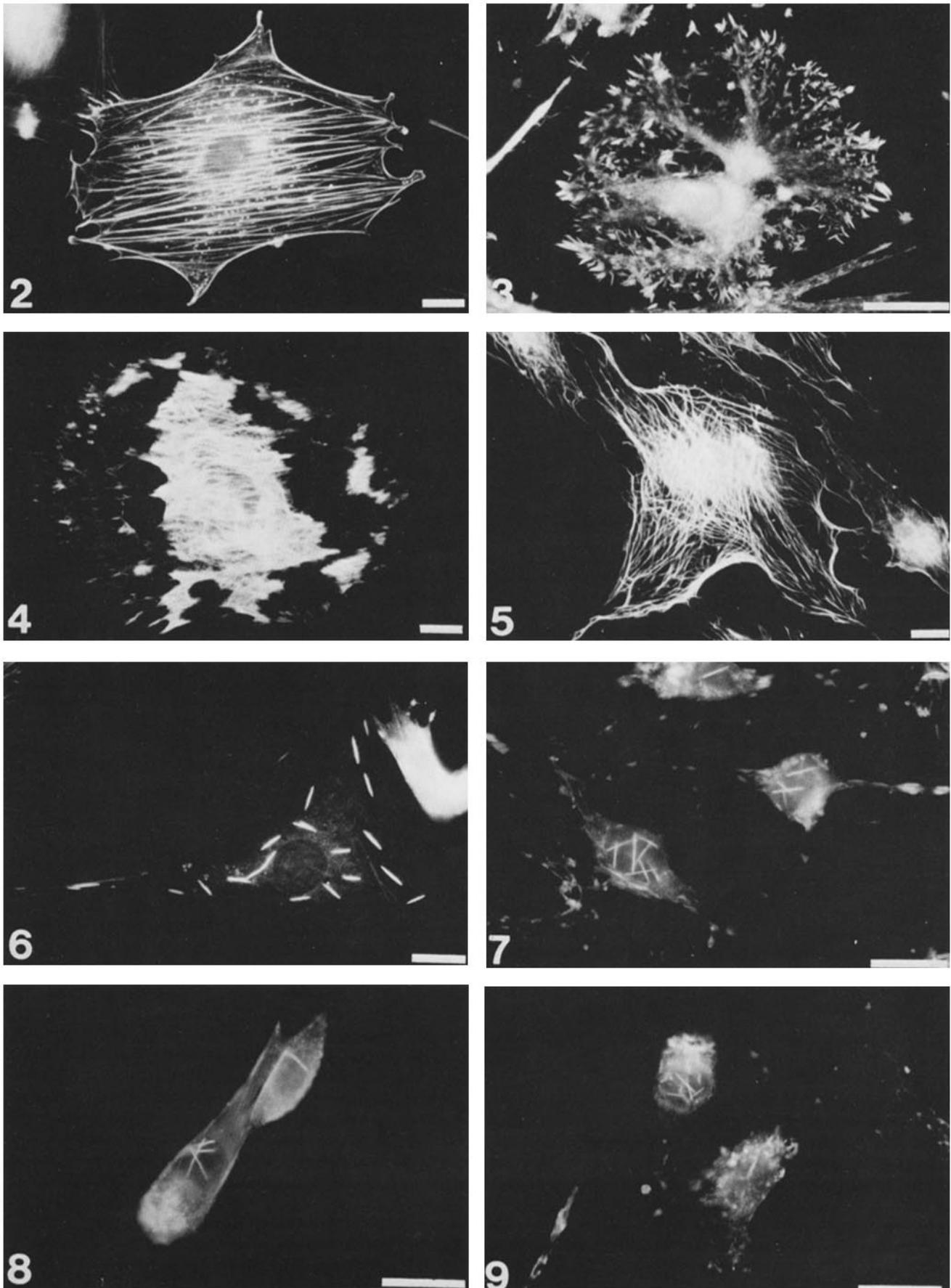


FIGURE 2-9 Immunofluorescence staining with actin antibody of mouse C3H cells untreated or treated with cytochalasins or with 10% DMSO at 37°C. Fig. 2: untreated cell.  $\times 360$ . Fig. 3: treated with  $20 \mu\text{M}$  CB for 1 h.  $\times 820$ . Fig. 4: treated with  $20 \mu\text{M}$  ChB for 30 min.  $\times 440$ . Fig. 5: treated with  $20 \mu\text{M}$  ChK for 30 min.  $\times 360$ . Fig. 6: treated with  $20 \mu\text{M}$  AsD for 30 min.  $\times 480$ . Fig. 7: treated with  $20 \mu\text{M}$  CD for 30 min.  $\times 720$ . Fig. 8: Incubated in 10% DMSO for 30 min.  $\times 720$ . Fig. 9: treated with  $20 \mu\text{M}$  CD for 30 min. CD was dissolved in ethanol in 2 mg/ml and was diluted with the medium.  $\times 720$ . Bars,  $20 \mu\text{m}$ .

decoration of the filaments within the rodlets with HMM could not be done.

Deoxaphomin, a 10-phenylcytochalasin, and CA were also found to induce the formation of cytoplasmic rodlets, but were less effective than AsD. Other cytochalasins tested in this study did not induce the formation of the rodlets in the cytoplasm.

Different from the cytoplasmic rodlets induced by AsD, CA or deoxaphomin, CB, CC, CD, and CH were found to induce the formation of rodlets in nuclei. CD was most effective in inducing the nuclear rodlets (Fig. 7 and 11). 10% (vol/vol) DMSO has been reported to induce the formation of nuclear rodlets consisting of actin filaments in a slime mold, *Dictyostelium* (14, 15) and other cultured cells (15, 38). We have also found that in C3H cells 5–10% DMSO induced similar nuclear rodlets to those seen in CD-treated cells (Fig. 8). Although the final concentration of DMSO in the incubation mixture con-

taining CD was 0.5% and this concentration of DMSO was known not to induce nuclear rodlets (data not shown), we have critically examined the possibility that the solvent in which CD was dissolved might cause the formation of nuclear rodlets. However, the nuclear rodlets were found also in cells treated with CD dissolved in ethanol (Fig. 9). The final concentration of ethanol was 0.5%. Ethanol even in 5% (vol/vol) did not induce the formation of nuclear rodlets. These results clearly suggest that CD is the cause of the rodlet formation. The CD-induced nuclear rodlets were decorated with HMM by the established method (20) (Fig. 12).

### Inhibition of Lymphocyte Capping

An effect on capping induced on mouse splenic lymphocytes with R-anti-MIg was determined using cytochalasins at 20  $\mu$ M

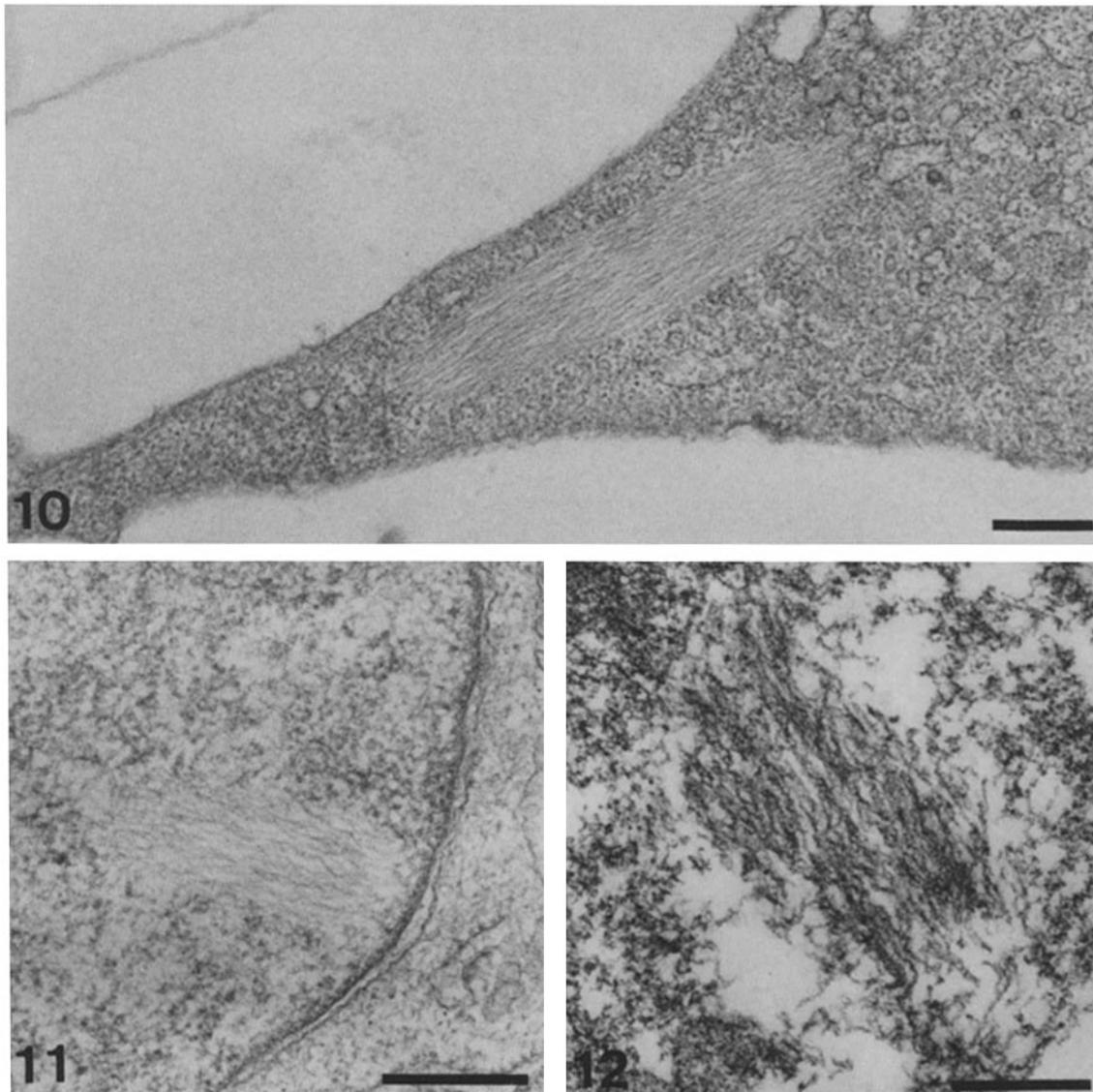


FIGURE 10 AsD-induced cytoplasmic rodlet consisting microfilaments. C3H cells were incubated with 20  $\mu$ M AsD at 37°C for 30 min. Thin sections for electron microscopy were prepared as described in the text. Bar, 0.5  $\mu$ m.  $\times$  26,700.

FIGURE 11 CD-induced nuclear rodlet consisting of microfilaments. C3H cells were incubated with 10  $\mu$ M CD at 37°C for 30 min. Bar, 0.5  $\mu$ m.  $\times$  40,200.

FIGURE 12 Decoration of filaments in CD-induced rodlet with HMM. C3H cells were incubated with 10  $\mu$ M CD at 37°C for 30 min, glycerinated, and incubated with HMM. Bar, 0.5  $\mu$ m.  $\times$  37,500.

(Table I, column 5). Lymphocytes were preincubated with cytochalasins at room temperature for 10 min, and then incubated with FITC-R-anti-MIg to induce capping. An extent to which capping was inhibited by the drugs varied, but appeared to be roughly proportional to the effect on membrane ruffling (Table I, column 2).

The viability of cells, determined by 0.04% trypsin blue exclusion, on which capping was inhibited by >90%, was >85%. Similar to the effect on cell morphology, an inhibition of capping by CA, ChA, ChD, ChJ, ChA-Ac, and ChD-Ac was found to be irreversible or slightly reversed within 10 min after the removal of the drugs from the incubation medium. The effects of other drugs were completely reversed during the same period of incubation. When the six cytochalasins causing the irreversible inhibition of capping at 20  $\mu$ M were used at 2  $\mu$ M, the inhibitions were almost fully reversed within 10 min. Furthermore, a prolonged incubation up to 1 h in an inhibitor-free medium restored to some extent capping of cells treated with these drugs at 20  $\mu$ M.

The inhibition of capping by cytochalasins was not affected by the presence or absence of 10 mM glucose in the medium. In addition, it should be noted that the degree of the inhibition is not proportional to the potencies in inhibiting sugar transport (34). These results strongly suggest that an inhibition of capping by cytochalasins can not be attributed to a possible deficiency in metabolic energy as a result of inhibition of glucose transport by the same drugs.

Ligand-independent capping induced in a hypertonic medium on mouse lymphocytes (49) was more severely inhibited by cytochalasins. For instance, ChJ at 2  $\mu$ M inhibited ligand-independent capping by 97%, but inhibited anti-Ig induced capping of the same cells by 44%. As has been shown (12) colchicine-treated lymphocytes were found to be more sensitive to the inhibitory effect of cytochalasins on capping induced by anti-Ig than were untreated cells. In the both systems, of ligand-independent capping and anti-Ig-induced capping on colchicine-treated cells, the tendency related to the degree of inhibition caused by various cytochalasins was found to be the same as that observed in the case of R-anti-MIg-induced on untreated cells (data not shown).

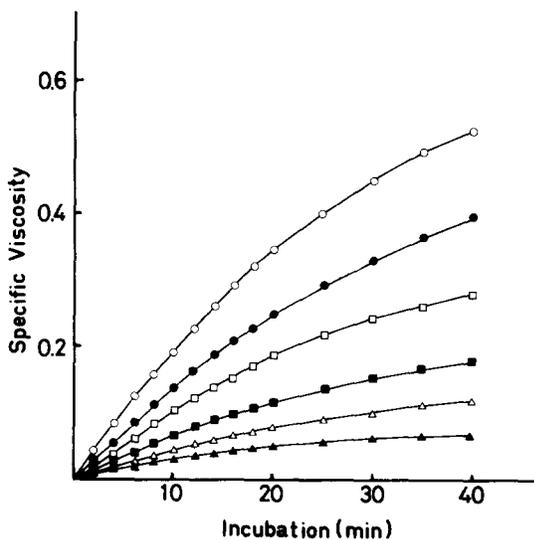


FIGURE 13 Effect of cytochalasins on actin filament elongation.  $\circ$ , control;  $\bullet$ , ChE;  $\square$ , CB;  $\blacksquare$ , CH;  $\triangle$ , ChJ; and  $\blacktriangle$ , ChA. The concentration of the drugs was 2  $\mu$ M.

### Inhibition of Actin Filament Elongation

To determine the high affinity effect of cytochalasins in vitro (8, 13, 29), we have determined effects of various cytochalasins at 2  $\mu$ M on actin filament elongation. Actin monomer (1 mg/ml) was induced to polymerize in buffer A by the addition of  $MgCl_2$  to 0.55 mM and a small amount of polymerized actin as nuclei for further polymerization. The polymerization, as measured by viscometry, started without any lag period, then proceeded almost linearly up to 20 min, and declined thereafter (Fig. 13). A slight increase in viscosity was still detected even 60 min after the start of polymerization (data not shown). An increase in specific viscosity per min, which was obtained on the average between 10 and 20 min, was  $0.0152 \text{ min}^{-1}$  for the control mixture with no inhibitor, and this value reveals the rate of actin filament elongation in this system.

Cytochalasin was added to the actin monomer solution before the initiation of the polymerization with  $MgCl_2$  and polymerized actin. The rate of actin filament elongation was reduced to different extents with different cytochalasins (Table I, and Fig. 13).

### Effect on Viscosity of Polymerized Actin

To assess the low affinity effect of cytochalasins in vitro (19), the effect of cytochalasins at 20  $\mu$ M on viscosity of polymerized actin was determined. To fully polymerized actin solution (1 mg/ml) in buffer A containing 2 mM  $MgCl_2$ , cytochalasin was added to 20  $\mu$ M. A change in viscosity was monitored up to 40 min, within which the decreasing reaction in viscosity did not reach the plateau with some cytochalasins, e.g., CE (19) and ChA. Percent decrease in specific viscosity of polymerized actin was determined at 40 min for each cytochalasin, and was used to show the low affinity effect of the drug (Table I, column 7, and Fig. 14).

As can be seen in Table I, columns 6 and 7, a cytochalasin, which show a strong effect via the high affinity binding sites, revealed also a strong effect via the low affinity binding sites. A correlation coefficient between the high affinity effect and the low affinity effect determined in vitro was calculated from these results to be 0.90 (Fig. 15).

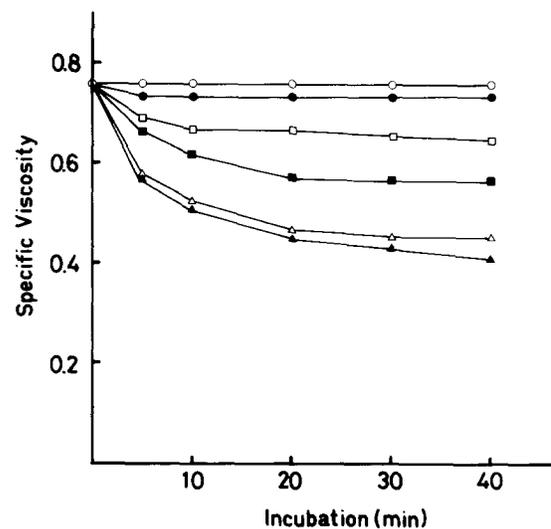


FIGURE 14 Effect of cytochalasins on viscosity of fully polymerized actin.  $\circ$ , control;  $\bullet$ , ChE;  $\square$ , CB;  $\blacksquare$ , CH;  $\triangle$ , ChJ; and  $\blacktriangle$ , ChA. The concentration of the drugs was 20  $\mu$ M.

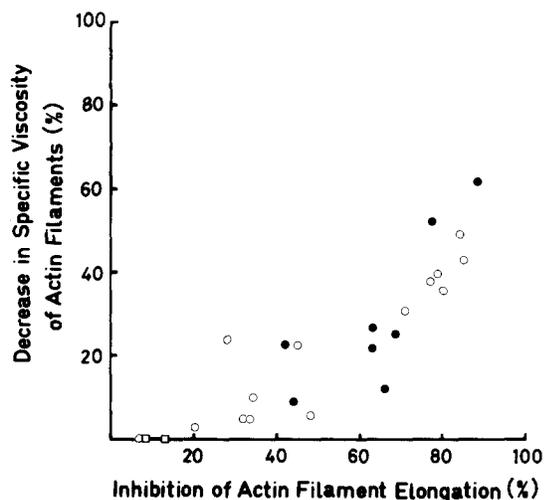


FIGURE 15 Correlation between two effects, inhibition of actin filament elongation and decrease in viscosity of polymerized actin, determined with each of 24 cytochalasins. Data shown in Table I, columns 6 and 7 were used. ●, 10-phenylcytochalasins; ○, 10-indolylcytochalasins; and □, 10-isopropylcytochalasins.

## DISCUSSION

### Low Dose and High Dose Effects

Low doses (0.1–1  $\mu\text{M}$ ) of CB have been reported to inhibit rapidly cell migration and membrane ruffling, but not to alter the gross morphology of fibroblastic cells (16, 42). In addition, high doses (5–20  $\mu\text{M}$ ) of the same drug induced gradually an alteration of cell morphology, resulting in arborization and rounding up of the treated cells (37, 46). The mechanisms of these two CB effects have been suggested to be different because low doses of CB disrupted microfilaments networks but do not result in obvious ultrastructural alterations in microfilaments bundles (16, 42), whereas high doses of CB caused a drastic rearrangement of microfilamentous structures (46). However, the results shown in Table I, columns 1 and 2, indicate that a drug which shows a strong low dose effect, such as the inhibition of membrane ruffling, also gives a strong high dose effect, the rounding up of cells. This seems to suggest that both of these effects caused by a cytochalasin might be attributed to the interaction between the same active site of the drug and the common cellular target. Different dose responses seen in the two effects may be explained by differential sensitivities to the cytochalasin of the structures that consist of the common target protein and are responsible for the cellular events in question.

However, there was an exceptional case that ChK, a 10-indolyl-cytochalasin bearing additional methyl groups at C-10 and -11 positions, did not induce rounding up of fibroblasts at 20  $\mu\text{M}$ , whereas the same drug of 0.2  $\mu\text{M}$  strongly inhibited membrane ruffling (Table I). Furthermore, ChK was found not to cause the contraction of actin cables while it inhibited capping. The absence of correlation between the high- and low-dose effects of ChK could not readily be explained. It has been shown that metabolic inhibitors such as 2-deoxyglucose or dinitrophenol interfered with the action of CB and CD (30), and the cell contraction was not induced with CB on glycerinated fibroblasts (46). Therefore, ChK may act on cells as a metabolic inhibitor or a membrane-disrupting agent besides a common cytochalasin. However, this remains to be proven.

### Cytochalasin-induced Rearrangement of Microfilamentous Structures

The contraction of actin cables is induced by all the cytochalasins used in this study, except ChK and ChE-Ac, an ineffective derivative of ChE (Table I) and is considered to be the initial stage of cell rounding up (reference 46, and this study). The contraction does not occur in glycerinated cells (46) and is inhibited by some metabolic inhibitors (30). Furthermore, the addition of ATP to glycerinated cells induces the cell contraction even in the presence of CB (46). These results suggested that ATP might be necessary for the cytochalasin-induced contraction. Whatever is the mechanism of the ATP-dependent contraction, cytochalasins trigger the contractions by altering the actin cables so that they respond to ATP and then contract.

It seems that there is no strict relation between an ability of a cytochalasin to induce hairy structures containing actin and another effect of the same drug. However, we may suggest that potent cytochalasins, as judged by abilities to induce cell rounding up and to inhibit rufflings, did not, or if anything, did weakly, induce the formation of hairy structures. This rule appears to hold particularly for the family of 10-indolyl-cytochalasins. It is possible that hairy structures could not be formed when actin-containing structures were disrupted to a large extent by potent cheatoglobosins such as ChA, ChD, or ChJ. This idea is supported by the fact that simultaneous addition of ChE at 20  $\mu\text{M}$  and ChA at 20  $\mu\text{M}$  did not induce the hairy structures (unpublished observations).

Among the cytochalasins used in our study, AsD, a 10-isopropyl-cytochalasin, is the most intriguing agent that revealed a property quite different from the others. That is the formation of cytoplasmic rodlets which appeared to be bundles of 50–80 Å microfilaments. Rathke et al. (35) have reported that CA induced similar rodlets in a clone of 3T3 fibroblasts. We found that AsD was more effective than CA in inducing the formation of cytoplasmic rodlets in C3H cells. Although detailed description of AsD-induced phenomena and an analysis of AsD effects will be published elsewhere (I. Yahara, F. Harada and W. Keller-Schielein, manuscript in preparation), we would make a few remarks concerning the formation of cytoplasmic rodlets. The ultrastructures of the rodlets are similar to those of nuclear rodlets which have been found on a variety of cells under various conditions (11, 14, 15, 19, 38). In addition to the structural similarity between AsD-induced cytoplasmic rodlets and nuclear rodlets, it was shown that neither contains  $\alpha$ -actin or tropomyosin (reference 38 and this study). The cytoplasmic rodlets were induced with 5–20  $\mu\text{M}$  AsD also on mouse embryonic fibroblasts and 3T3 cells, but not on HeLa or SV3T3 cells (unpublished results). Differential effects of AsD on different cell types are under investigation.

We have found that some cytochalasins, including CD, induced nuclear rodlets. We have excluded the possibility that the formation of nuclear rodlets was induced by the solvent in which CD or other drugs had been dissolved. The fact that both CD and 10% DMSO induced the same rodlet formations and that there is no similarity in molecular structure between CD and DMSO suggest that neither CD nor DMSO molecules might be directly involved in the mechanism of rodlet formation. Instead, CD or DMSO may alter cellular conditions including the supramolecular assembly and topography of microfilaments so that these filaments assembled into the rodlets. In this regard, it is of interest that actin is transferred from the cytoplasm to the nucleus in DMSO-treated cells (38).

## Correlation between Effects of Cytochalasins on Cellular and Molecular Levels

As shown in Table I (columns 1, 2, and 5), we have assessed potencies of 24 cytochalasins to affect cellular events. The results suggest that the extent of the effects is independent of the assay method and the cell types used in the experiments. To compare the cellular and molecular effects for each cytochalasin, an inhibition of cap formation was used because it was the only cellular effect that was expressed numerically. Fig. 16 was obtained by plotting an inhibition (%) of capping vs. that of actin filament elongation (Table I, column 6) for each cytochalasin. A correlation coefficient ( $r$ ) between the two effects was calculated to be 0.87 from data obtained with 24 cytochalasins. Similarly,  $r$  between the effect on capping and that on viscosity of actin filaments (Table I, column 7) was obtained to be 0.79. The positive correlation between effects on the molecular and cellular levels strongly suggests that an extent to which cellular structures or events are affected by a cytochalasin is specifically determined by its ability to affect actin molecules in vitro.

There may be other factors, e.g., permeability through the plasma membrane or modification of cytochalasin molecules inside the cells, determining the magnitude of the cellular effects, but the results suggested that they must be trivial if any. For instance, the correlation suggests that the inability of ChE-Ac to affect cellular events is not due to a possible low permeability of the drug. It might be noted that values for inhibition of actin polymerization by 10-indolylcytochalasins, indicated by open circles, are slightly lower than those by 10-phenylcytochalasins, indicated by closed circles, when these effects on capping are approximately the same. This difference may be explained by different permeabilities of these two families of cytochalasins.

The results obtained in this study are in good agreement with the previous observations by others (9, 19, 26, 29) that four cytochalasins, CB, CD, CE, and dihydro-CB, inhibited actin polymerization in vitro, and the relative magnitudes of these effects correspond to their potencies in affecting cell motility.

By viscometric (8, 9, 13, 29) and electron microscope (29)

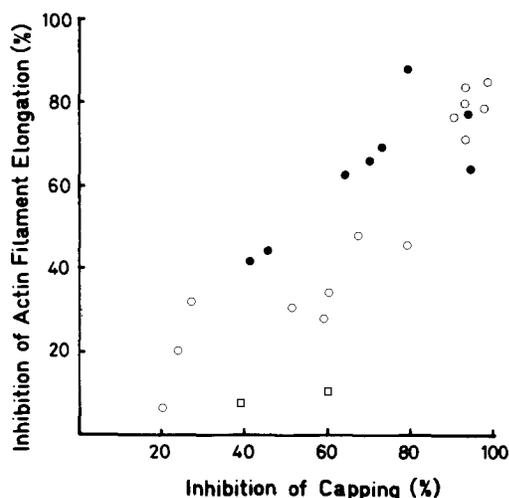


FIGURE 16 Correlation between inhibition of capping and inhibition of actin filament elongation by cytochalasins. Percent inhibition of capping was plotted vs. percent inhibition of actin filament elongation for each of 24 cytochalasins. Data shown in Table I, columns 5 and 6 were used. Symbols are same as those used in Fig. 15.

analyses on the affect of CB, CD, and CE at relatively low doses on purified actin polymerization and by measurement of CB binding to filamentous actin (9), it has been recently suggested that cytochalasins interact with the end of filamentous actin so that the elongation of the filaments is inhibited. In addition to this low dose effect of cytochalasins, submicromolar CB inhibits network formation by pure actin filaments (18, 29). Furthermore, cytochalasins at high doses decrease viscosity of polymerized actin through an interaction with the low affinity sites (reference 19, Fig. 14, and Table I, column 7). Therefore, the molecular mechanism of cytochalasin actions on the cellular level may not be unique, although the target protein of cytochalasins in cells is always actin.

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