Mini-Review

Effects of Cytochalasin and Phalloidin on Actin

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Cytochalasins and phalloidins are two groups of small, naturally occurring organic molecules that bind to actin and alter its polymerization. They have been widely used to study the role of actin in biological processes and as models for actin-binding proteins. Functionally, cytochalasins resemble capping proteins, which block an end of actin filaments, nucleate polymerization, and shorten filaments. No known actin-binding protein stabilizes actin filaments as phalloidin does, but such proteins may have been missed. Cytochalasin and phalloidin have also helped to elucidate fundamental aspects of actin polymerization. This review briefly summarizes older studies and concentrates on recent work on the mechanisms of action of cytochalasin and phalloidin.

Cytochalasin

Cytochalasins, a group of fungal metabolites, permeate cell membranes and cause cells to stop ruffling and translocating, round up (44, 54), become less stiff (12), and enucleate (28). In addition to binding actin, cytochalasins A and B also inhibit monosaccharide transport across the plasma membrane; however, cytochalasins C, D, E, H, and 21,22-dihydrocytochalasin B do not (42).

Cytochalasin Binding to Actin Filaments. Cytochalasins bind to the barbed end of actin filaments, which inhibits both the association and dissociation of subunits at that end. The stoichiometry of binding is about one cytochalasin per actin filament (8, 19); in these studies the filament number could not be determined accurately. Measurements of the affinity, based on different types of experiments, are compiled in Table I. The dissociation constant for binding (K_d) is determined with radiolabeled cytochalasin and characterizes the structural interaction between cytochalasin and actin. The inhibition constant (K_i) is measured from the effect of cytochalasins on the growth or shortening of the barbed end of actin filaments. CD[1] is about 10 times more effective than CB. For both cytochalasins, the binding and inhibition constants agree fairly well, which shows that binding causes inhibition of polymerization and depolymerization.

The inhibition constant for growth with ATP-actin is quite different from the others and varies with the actin monomer concentration (9). These complications are probably attributable to the state of the nucleotide in the different experiments. The binding studies are performed with ATP-actin at steady state, where no net growth or shortening of filaments occurs. Actin monomers have mainly bound ATP, and filaments have mainly ADP because the ATP hydrolyzes after the monomer adds to the filament. In the functional studies in ADP all of the actin molecules have bound ADP. In ATP, free monomers will have bound ATP but the ends of the filaments can have either ATP, ADP, or a mixture of both, depending on the relative rates of subunit addition and ATP hydrolysis. In experiments where the constants agree, the filament ends probably have bound ADP. Since the plot of apparent K_i vs. ATP–actin monomer concentration has a positive curvature, CD may not bind at all to filaments with ATP-actin ends, so that the variable effect of CD may simply reflect the proportion of ADP-actin ends (9). Alternatively, CD may induce dimerization of ATP-actin monomers, as discussed in detail below. New experiments are needed to measure the binding affinity for ATP-actin filaments, but the short lifetime of these filaments makes this technically difficult.

Electron microscopy of filaments grown from morphologically identifiable seeds has revealed that the major effect of cytochalasin is at the barbed, as opposed to the pointed, end (4, 36). In a recent set of experiments 2 μM CB inhibited association and dissociation events only by 90%, and 2 μM CD had a similar effect (4). This interesting result should be confirmed by showing that the dependence of barbed end elongation on CD concentration exhibits a plateau at 90% instead of 100% inhibition.

The rate constants for cytochalasin binding to barbed ends are of interest but have not been measured. If the 90% inhibition by 2 μM CB is due to 90% binding, then the rates of association and dissociation of CB must be at least compara-

Table I. Apparent Equilibrium Dissociation Constants of Binding and Inhibition for Cytochalasins and Actin Filaments

	K_d	K_i			
		ADP-Actin		ATP-Actin	
	Binding	Filament growth	Filament shortening	Filament growth	Filament shortening
	nM	*nM*	*nM*	*nM*	*nM*
CB	5–40	40	40	200	–
CD	∼2	1–2	1–2	2–35+	2

The methods are discussed briefly in the text. The values are taken from the following references: K_d for CB, 8 and 19; K_d for CD, 19; K_i for ADP-actin for CB, 7; K_i for ADP-actin for CD, 9; K_i for ATP-actin growth for CB, 7 and 19; K_i for ATP-actin growth for CD, 9 and 19; K_i for ATP-actin shortening for CD, 9.

1. *Abbreviations used in this paper*: CB, cytochalasin B; CD, cytochalasin D.

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ble to the rates of association and dissociation of actin monomers, since filaments grow uniformly with time (4).

Some (14, 30), but not all (36), studies find that cytochalasin shortens actin filaments. The mechanism for shortening is unresolved because we lack both methods to measure filament length and theories that combine all the factors that affect filament length. One interesting possibility is that cytochalasin can bind to a subunit in the interior of an actin filament and break the filament in two, called "severing." An electron microscope assay for CB does not show the dramatic shortening of filaments characteristic of severing by certain capping proteins (3, 29). Nevertheless, interruptions of long filaments are seen, which may represent capping of transient filament breaks induced by shear during the final stages of sample preparation (4).

Cytochalasin Binding to Actin Monomers and Dimers. Goddette and Frieden recently examined the binding of CD to monomeric actin and the formation of actin dimers, inspired by the observations that cytochalasin increases the rate of spontaneous polymerization of actin monomers (45) and the rate of ATP hydrolysis by actin monomers (6). The direct binding of CD to monomeric actin was measured with a sensitive assay for free CD (24). In a nonpolymerizing buffer with Ca^{++}, the K_d is 18 μM, and the stoichiometry is 1:1. When Mg^{++} replaces Ca^{++}, the K_d is 2.6 μM but the stoichiometry is strikingly unexpected: one CD per two actins, which suggests either that actin dimerizes or that half of the actin monomers are incapable of binding CD. Direct physical evidence for dimer formation was obtained by small angle neutron scattering (27). Without CD a moderate amount of dimers form over several hours in the presence of Mg^{++}. In CD, dimers form more rapidly (on a time scale consistent with the previous binding studies) and to a greater extent.

The rapid kinetics of cytochalasin binding and dimer formation were studied using actin labeled with fluorescent probes. AEDANS-actin was used to measure the binding of CD to monomeric actin (25). The fluorescence of AEDANS-actin is higher with bound Mg^{++} than Ca^{++}, and addition of CD to Mg^{++}-actin leads to a decrease in fluorescence. The time course of the fluorescence decrease was monitored with stopped-flow techniques, as a function of the concentrations of CD, actin, and Ca^{++}. Changing the actin concentration has no effect, and so the fluorescence decrease is not due to dimerization. Taken together, the data are consistent with a theoretical model in which CD binds rapidly and loosely to an actin monomer, followed by a conformational change of the complex to a state where the CD is bound more tightly (25).

Pyrene-actin was used to study the kinetics of dimer formation (26). Pyrene-actin cannot bind CD directly, hence fluorescence changes represent the formation of dimers or larger oligomers. The fluorescence of pyrene-actin filaments is 20 times that of monomers, and the time course of spontaneous polymerization of monomers, in the absence of CD, has a characteristic sigmoidal shape, reflecting slow nucleation and subsequent rapid elongation. In the presence of CD, several changes occur in the time course of fluorescence. The most obvious ones are that the lag phase is eliminated, interpreted as accelerated nucleation, and that the final steady-state fluorescence is decreased, which implies a higher critical monomer concentration (45).

Two subtle, rapid changes in fluorescence provide information about how actin dimers may form and act. Upon addition of CD and Mg^{++}, the fluorescence of pyrene-actin increases rapidly and then partially decreases more slowly (26). The magnitudes of these changes are relatively small, and the changes occur before the large increase in fluorescence attributable to filament formation. The initial fluorescence increase is hypothesized to represent dimer formation. The kinetics of this rapid initial increase are predicted by a model in which CD and Mg^{++} bind to actin, induce dimer formation, and the dimer undergoes a conformation change to the high fluorescence state (26). The dependence of the steady-state pyrene-actin fluorescence on the total actin concentration provides additional evidence for the existence of oligomers induced by CD. In the presence of CD, plots of fluorescence vs. actin concentration are curved, in contrast to controls that have a characteristic sharp transition at the critical concentration (9).

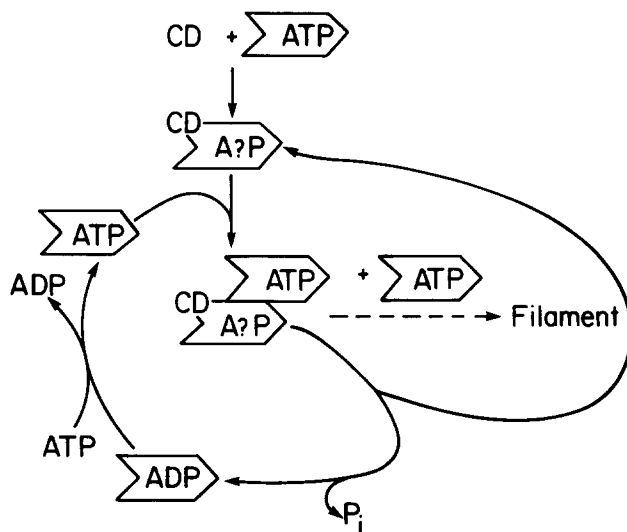

Figure 1. A model for the interaction of CD with actin monomers and dimers, described by Goddette and Frieden (26). The chevron symbol represents an actin monomer. A?P denotes uncertainty about whether the bound nucleotide is ATP or ADP. The wavy line leading to filament represents nucleation. The model does not specify whether the nucleating species is a CD-actin dimer with ATP or ADP. This uncertainty is not depicted.

Goddette and Frieden (26) propose a model that qualitatively explains these new observations along with several important older pieces of data (Fig. 1). When CD-actin dimers form, the actin contains bound ATP. The ATP is hydrolyzed to ADP, which causes the dimers to fall apart, generating a CD–actin monomer complex and a free actin with ADP. The CD-actin dimers are good nucleators, but CD-actin monomers are poor nucleators. A CD-actin monomer can bind an ATP-actin monomer to re-form a CD-actin dimer. The cycle repeats with hydrolysis of ATP and creation of an ADP-monomer.

How does this model explain the data? (*a*) Dimer formation and dissociation explain the increase and subsequent decrease of pyrene-actin fluorescence (26). CD-actin dimers form in high concentration transiently because the molecules proceed synchronously through the first turnover of the cycle.

(*b*) ATP is hydrolyzed by monomers and dimers, without the involvement of filaments, which explains the increased

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rate of ATP hydrolysis by actin monomers in cytochalasin (6). Also, the Mg^{++} dependence of dimer formation and polymerization predicts the Mg^{++} dependence of the ATPase. In the absence of Mg^{++}, CD does not cause dimer formation or increase the ATPase. 100–500 μM Mg^{++} suffices to form dimers (26) and increase the ATPase (6, 34). Greater than 1 mM Mg^{++} causes actin polymerization, and so monomers incorporate into filaments rather than form dimers. The model therefore predicts a lower ATPase, as observed by Low and Dancker (34).

(*c*) Cytochalasin is a poor nucleating agent, which is explained in the model by some dimers dissociating instead of proceeding through nucleation. One might not need to invoke dissociation to explain why CD-actin dimers are poor nucleators. CD-actin dimers should be poor nucleators because they nucleate growth of actin filaments only in the pointed direction (CD should cap the barbed end of the new filament). Although this consideration explains why CD-actin dimers are worse nucleators than actin dimers without CD, it does not explain why a given concentration of plasma gelsolin, which also nucleates growth in the pointed direction, nucleates better than the same concentration of CD (45). Plasma gelsolin probably binds actin monomers more tightly than does CD (13), and so fewer dimers may form in CD than in gelsolin, which would account for the poor nucleating activity of CD. Also, CD-actin dimers may be structurally different from other dimers and thereby poor nucleators. To resolve this issue properly, one would like quantitative measurements of the concentration of CD-actin dimers and the fraction of dimers that dissociate or nucleate filament formation.

(*d*) In CD the apparent critical concentration for polymerization is high. One expects a higher critical concentration simply because the barbed ends are capped (the pointed end has a higher critical concentration than the barbed end in Mg^{++} (2)). Goddette and Frieden (26) find that the apparent critical concentration in CD is higher than that of the pointed end. Their model explains this difference qualitatively by the formation of ADP-actin monomers, which have a high critical concentration (8 μM under these conditions) (41). Although the ADP will exchange with free ATP in solution, the exchange may be slow enough that ADP-actin persists in appreciable concentrations. Slow nucleotide exchange can theoretically predict a relatively high ADP-actin monomer concentration for actin filament solutions at steady state (39), and can explain the increase in critical concentration for sonicated actin (41). The precise values of the on and off rate constants for nucleotides binding to actin monomers are not yet known but would permit a quantitative test of the model.

The literature, however, disagrees as to whether the apparent critical concentration in CD really is higher than that of the pointed end (measured as the apparent critical concentration in plasma gelsolin). Results from Korn's laboratory show the same apparent critical concentration (4 μM) in CD and plasma gelsolin (9, 13), but results from Frieden's laboratory show a difference (26, 45). The experimental protocols of the two laboratories differ in two major respects: (*a*) the actin is either monomeric or is prepolymerized to filaments at time zero, and (*b*) the time of incubation varies from 4 to 24 h. To understand this difference, we performed an experiment that compared the different conditions (Table II) (K. Patane, J. A. Cooper, and C. Frieden, unpublished results). At 24 h the data for filament and monomers are the same and the difference between CD and plasma gelsolin exists.

Table II. Comparison of the Apparent Critical Concentration for Actin Polymerization in CD and Plasma Gelsolin

	Physical State of Actin at Time Zero			
	Monomer		Filament	
	4 h	24 h	4 h	24 h
	μM	*μM*	*μM*	*μM*
CD	5.9	5.1	4.3	5.1
Plasma Gelsolin	2.1	2.1	3.8	2.6

Actin was incubated with CD at 0–5 μM or plasma gelsolin at 0–1 μM. The apparent critical concentration was calculated from the pyrene-actin fluorescence at each concentration of CD and plasma gelsolin. The values in the table are from the plateau portion of the curve, which was at 5 μM CD and 0.5 μM plasma gelsolin. Based on previous results, we assume that actin alone polymerizes to steady state in 4 h with a critical concentration of 0.5 μM. Conditions: 12 μM rabbit skeletal muscle actin (2% pyrene-labeled), 1 mM $MgSO_4$, 2 mM Tris/HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 20°C. When an additional 0.2 mM ATP was added after the measurements at 24 h, the fluorescence did not change.

Alternatively, the high apparent critical concentration in CD could be due to CD-actin monomers that do not polymerize. An experiment that includes a range of actin concentrations, however, would reflect this contribution with a characteristic shape in the plot of fluorescence vs. actin concentration, as described for *Acanthamoeba* profilin (33). This shape is not seen with CD (9).

In the future, the mechanism of action of cytochalasin on actin can be tested and elucidated with new experiments and complex modeling. New experiments are needed to address issues such as the rate of nucleotide exchange in Mg^{++} and CD because the slow release of ADP from monomers is a key feature of the model. Also, other measurements of the physical state of the actin would be important to confirm that the fluorescent probes accurately report the state of assembly. Testing the internal consistency of the complete mechanism with computer-assisted simulation will eventually be desirable when there is a complete set of data for one condition.

Effects of Cytochalasin on Cells. To understand the role of actin in cell motility, one would like probes that are specific for actin and affect only one aspect of actin's polymerization or interaction with other proteins. Although cytochalasins are the best available probes, they do not satisfy these criteria fully.

Cytochalasin D is probably specific for actin. Although the possibility of CD having targets other than actin cannot be totally excluded, three kinds of evidence argue for specificity. First, CD does not bind to the glucose transporter, as do some cytochalasins, including CB (42), which should never be used to study cell motility. Binding of CD to other targets has not been reported. Of concern, however, are the observations that CD inhibits protein synthesis (40) and alters the impedance of membranes (43). Second, the affinity of CD for barbed ends is high (K_d 2 nM), so it can be used in low concentrations to minimize nonspecific interactions. Despite this fact most experiments have used high concentrations (2 μM). Third, other probes that interact with ac-

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tin have similar effects on cells. Microinjection of several unrelated capping proteins causes the same morphologic and functional effects on cells as CD (12, 22). The chance that all these probes have a mechanism other than actin must be quite low.

If one grants that CD is specific, then an experiment where CD alters a cellular process implies that actin has some role in that process. This conclusion, although valuable, is limited because of the multiple effects of CD on actin. The dependence of a cellular process on the CD concentration may distinguish its effects on barbed ends and monomers. The rate of permeation of CD across the plasma membrane and the rate of the cellular response must be rapid if such an experiment is to yield conclusive results.

This approach has been used in some experiments. Low concentrations (0.2 μM) of CD inhibit membrane ruffling, which implicates growth or shortening of barbed ends (54). The peripheral area of cells, where ruffles begin, contains actin filaments that are rapidly growing and shortening in a "treadmilling" fashion (48). The treadmilling is constitutive, but ruffles only occur at certain places and times. Regulatory elements may control where and when treadmilling causes a ruffle to form. Higher concentrations (2–20 μM) are necessary to remove stress fibers (54), which implicates CD as binding to monomers. Perhaps nucleation of new filaments removes actin subunits from stress fibers by mass action. The use of metabolic inhibitors prevents this effect (44). Since CD binding to actin monomers and nucleating filament formation probably depends on ATP (21, 24), the effect of metabolic inhibitors may be to lower the ATP concentration and prevent those processes. Alternatively, loss of ATP may put the actomyosin in stress fibers into rigor, which decreases the rate at which actin subunits or filaments leave.

On the other hand, several uncertainties about the in vitro mechanism limit the interpretation of experiments with cells. First, CD may not bind at all to barbed ends with ATP caps (9). While ATP caps might not exist at steady state, they may exist when a filament grows rapidly, which can occur in cells (46, 48). Second, the K_d for monomer binding was determined in low ionic strength and low Mg^{++} concentrations, so the K_d in cells may be different. Third, cells have high concentrations (100 μM) of nonfilamentous actin (5), most of which is probably bound to profilin or other proteins. Interactions of CD with this actin pool are unknown.

Even if monomer and filament binding can be distinguished by cytochalasin concentration dependence, each type of binding has several inseparable effects on actin polymerization. Capping barbed ends will inhibit both growth and shortening of filaments, and it will also increase the critical concentration since barbed and pointed ends probably have different critical concentrations in cells. Monomer binding leads to nucleation of filament formation as well as a higher critical concentration (Fig. 1). Another complicating factor is that cytochalasin may compete with cellular capping proteins for barbed ends. One can argue that the barbed end of all actin filaments in cells must be capped, otherwise the ends will constantly depolymerize (32). Since CD would competitively inhibit the binding of capping proteins to barbed ends, its effects may represent the loss of a capping protein that specifies the location or function of the filament.

In the face of all this uncertainty, what can one say about what cytochalasin does to actin in cells? The surest conclusion is that CD caps barbed ends. Cytochalasin inhibits growth of actin filaments in two model systems (16, 46). In these systems, the pointed ends are probably capped, and the barbed ends grow, although this point is not proven. The widely held idea that cytochalasin depolymerizes actin filaments is certainly not true in general. One expects a slight increase in critical concentration due to capping barbed ends and dimer formation (Fig. 1), but the quantity of this increase is only a few percent of the amount of the actin filaments. In fibroblasts, cytochalasin causes no change in the ratio of filamentous to nonfilamentous actin (38). Cytochalasin does prevent or reverse the increase in filamentous actin that accompanies platelet activation, but it does not decrease the filamentous actin in resting platelets (10, 20). Cytochalasin disrupts the supramolecular organization of actin filaments, but the relation of this phenomenon to the in vitro mechanism is unclear. Electron microscopy shows that actin filaments persist in cytochalasin; their organization changes from an isotropic network to focal accumulations (44). Severing actin filaments might explain this transition, but the severing activity of cytochalasin is weak (4). Alternatively, competition of cytochalasin with capping proteins for barbed ends may cause this change. If actin filaments are normally held in place by capping proteins that bind to their barbed ends, cytochalasin may release the filaments and allow them to be contracted into foci. The observation that stress fibers sometimes contract in cytochalasin, as though their membrane attachments were lost (54), also supports this hypothesis.

Phalloidin

Phallotoxins are a group of bicyclic heptapeptides from poisonous mushrooms (51). The major representative of this group, phalloidin, binds to actin filaments much more tightly than to actin monomers (17) and shifts the equilibrium between filaments and monomers toward filaments, lowering the critical concentration for polymerization by 10- to 30-fold under various conditions (17, 18). The lower critical concentration is due to a decrease in the rate constant for the dissociation of actin subunits from filament ends (11, 17). The dissociation rate constants at both the barbed and pointed ends are lower than the error in the measurement ($0.01\ s^{-1}$), so the actual magnitude of the change is uncertain but consistent with the effect on the critical concentration. The association rate constant at the pointed end does not change, but at the barbed end it decreases (the opposite of what is expected for a critical concentration decrease) by 20% (11).

For filaments the stoichiometry of binding is one phalloidin for either one or two actin protomers. A 1:1 value was inferred from the amount of phalloidin needed to protect actin filaments against depolymerization (15) and binding measured by difference spectroscopy (52). Another group found that a 1:2 ratio was sufficient to provide maximal protection against depolymerization, and in a pelleting assay with Scatchard analysis the stoichiometry was 1:1.7 with a K_d of 85 nM (37). This difference is difficult to resolve; the designs of the two sets of experiments are different, and the extinction coefficients and purity of the phalloidin and actin may be different. In experiments measuring the binding of radioactive phalloidin to liver plasma membranes, which probably reflects binding to actin filaments, a high affinity site of 22 nM was found. By displacement, the dissociation

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rate constant was 3.8×10^{-3} s^{-1}, and the association rate constant was calculated to be 0.17 μM^{-1} s^{-1} (35). Photoactivatable derivatives of phalloidin, bound to actin filaments, react covalently with amino acids Glu-117, Met-119, and Met-355 (47), which are very close to the nucleotide binding site (1).

Fluorescent derivatives of phalloidin have been extremely useful for localizing actin filaments in living and fixed cells (50, 53) and visualizing individual actin filaments in vitro (55). If saturating quantities of fluorescent phalloidin are used, then the fluorescence is a quantitative measure of the amount of filamentous actin in cells. In this approach, the fluorescence of single cells is measured with a fluorescence-activated cell sorter, or the fluorescence of a methanol extract of a group of cells is measured with a fluorometer (31).

The effects of phalloidin on actin are easy to interpret: it should prevent filament depolymerization and shift the equilibrium from monomer toward filament. Phalloidins, however, do not permeate cell membranes and have therefore not been very useful in experiments with living cells. They are taken up by many cells, probably by pinocytosis, and are avidly taken up by hepatocytes by an unknown mechanism (51). Cells treated with phalloidins show a variety of toxic effects and often die. While this toxicity could be mediated by actin, it raises the question of whether phalloidin has other targets, since cells treated with CD do not die. Phalloidin-treated cells have increased amounts of actin associated with their plasma membranes (23), and the microinjection of phalloidin into living cells alters actin distribution and cell motility (49).

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