RAPID COMMUNICATIONS

CYTOCHALASIN INHIBITS THE RATE OF ELONGATION OF ACTIN FILAMENT FRAGMENTS

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ABSTRACT

Submicromolar concentrations of cytochalasin inhibit the rate of assembly of highly purified *Dictyostelium discoideum* actin, using a cytochalasin concentration range in which the final extent of assembly is minimally affected. Cytochalasin D is a more effective inhibitor than cytochalasin B, which is in keeping with the effects that have been reported on cell motility and with binding to a class of high-affinity binding sites from human erythrocyte membranes (Lin and Lin. 1978. *J. Biol. Chem.* **253**:1415; Lin and Lin. 1979. *Proc. Natl. Acad. Sci. U. S. A.* **76**:2345); 5×10^{-7} M cytochalasin B lowers the rate to 70% of the control value, whereas 10^{-7} M cytochalasin D lowers it to 25%. Fragments of F-actin were used to increase the rate of assembly fivefold by providing more filament ends on to which monomers could add. Under these conditions, cytochalasin has an even more dramatic effect on the assembly rate; the concentrations of cytochalasin B and cytochalasin D required for half-maximal inhibition are 2×10^{-7} M and 10^{-8} M, respectively. The assembly rate is most sensitive to cytochalasin when actin assembly is carried out in the absence of ATP (with 3 mM ADP present to stabilize the actin). In this case, the concentrations of cytochalasin B and cytochalasin D required for half-maximal inhibition are 4×10^{-8} M and 1×10^{-9} M, respectively. A Scatchard plot has been obtained using [^{3}H]cytochalasin B binding to F-actin in the absence of ATP. The K_d from this plot ($\sim 4 \times 10^{-8}$ M) agrees well with the concentration of cytochalasin B required for half-maximal inhibition of the rate of assembly under these conditions. The number of cytochalasin binding sites is roughly one per F-actin filament, suggesting that cytochalasin has a specific action on actin filament ends.

KEY WORDS cytochalasin · actin assembly

The mechanism of action of cytochalasin remains unknown despite its extensive use in studies of cell shape and motility (for review, see reference 20). Many of these studies of effects on motility are complicated by the fact that cytochalasin concentrations in the micromolar to millimolar range were used. There is thus the possibility of nonspecific low-affinity binding to many sites, and con-

sequently little hope of correlating these effects with a molecular mechanism. If a high affinity effect can be demonstrated, then binding should be restricted to one or a few specific sites, and effects on motility may be interpretable. In fact, many effects on cell shape and motility have been reported at submicromolar cytochalasin. Also encouraging are the findings (2, 7, 13) that there is a hierarchy of effectiveness of different cytochalasins on motility-related functions: cytochalasin E

(CE) > cytochalasin D (CD) > cytochalasin B (CB). Any study of the mechanism of action of cytochalasin must satisfy these criteria of high-affinity binding and the hierarchy of effectiveness.

Previous investigations have revealed high-affinity binding to a sugar transport site in erythrocytes (10, 11). However, this binding cannot be linked to the motility-related effects, since among other things it does not show the order of effectiveness mentioned above.

More recently, high-affinity binding not related to glucose transport has been reported (5, 9). Furthermore, Lin and Lin (6) have partially purified a complex from erythrocyte membranes that binds cytochalasin with high affinity. This complex consists of several proteins, including actin. The binding of cytochalasin to this complex seems quite relevant to motility, since, the affinities of binding of various cytochalasins are in the same order as their relative potency in affecting cell morphology and motility. Furthermore, cytochalasin blocks the ability of this complex to nucleate actin assembly.

Here we report high-affinity binding of cytochalasin to highly purified *Dictyostelium* F-actin, possibly to the filament ends. This binding results in inhibition of the rate of assembly of G-actin into its filamentous state.

MATERIALS AND METHODS

Actin was purified from *Dictyostelium discoideum* by the method of Uyemura et al. (21) which included Sephadex G-150 and DEAE chromatography, and ammonium sulfate fractionation. Actin was depolymerized by sonication of an F-actin pellet (2 × 15 s, setting 4, Kontes ultrasonic cell disrupter, Kontes Co., Vineland, N. J.) into a buffer containing 3 mM imidazole, pH 7.5, 0.2 mM DTT, 0.1 mM ATP or 3 mM ADP, and allowed to incubate on ice for a minimum of 1 h. Polymerization of 0.2 mg/ml G-actin was initiated at 25°C by adding salts to 0.1 M KCl and 0.1 mM MgCl₂. Cytochalasin was added just before the addition of salts in a constant volume of dimethyl sulfoxide (DMSO) so that the final DMSO concentration was 0.5%. Control samples contained 0.5% DMSO without cytochalasin. Preincubations of G-actin with cytochalasin before the addition of salt had no effect on the results.

Filament fragments were obtained by sonicating (5 s, setting 4, Kontes ultrasonic cell disrupter) a sample of F-actin, prepared as described above in the absence of cytochalasin. Sonication caused a decrease in the reduced viscosity of the F-actin from 11 dl/g to ~2 dl/g. Immediately after sonication, 1 vol of 0.2 mg/ml fragments was added to 20 vol of 0.2 mg/ml G-actin ± cytochalasin, then immediately salts were added to initiate polymerization.

Actin concentration was determined by absorbance at 290 nm (minus that at 320 nm) with an extinction coefficient of 0.62 cm⁻¹·ml·mg⁻¹ (4) and by the method of Bradford (1).

Viscometry was performed at 25°C in a Cannon-Manning 100 semi-micro viscometer (Cannon Instrument Co., State Col-

lege, Penn.) (buffer flow time = 62 s) to follow the rate of assembly of actin at 0.2 mg/ml. Rate of assembly was calculated from curves such as those shown in Fig. 2 A, as follows: The final extent of assembly could be measured when the assembly rate was reasonably fast (see Table I); however, for very slow assembly, it was impractical to measure the final extent of assembly. Therefore, the rate in all cases has been defined as the time required to reach a reduced viscosity of 5.5 dl/g (one-half of the final reduced viscosity in the control reaction in the absence of cytochalasin).

Equilibrium dialysis was performed at 3°C with a Microvolume equilibrium dialyzer (Hoefer Scientific Instruments, San Francisco, Calif.) with 0.25-ml wells. "Gel backing" (Dialysis membrane from Biorad Laboratories, Richmond, Calif.) was boiled in 2 mM EDTA before use. One well was filled with 0.2 ml of F-actin (6–9 mg/ml) in 3 mM imidazole, pH 7.5, 0.2 mM DTT, 3 mM ADP, 0.1 M KCl, and 0.1 mM MgCl₂; the neighboring well was filled with 0.2 ml of [³H]CB (4 × 10⁻⁹ to 1.3 × 10⁻⁷ M) in the same buffer. Glass beads were added to the chambers to facilitate mixing as they rotated. Dialysis was carried out for 21–28 h at 3°C; dialysis for an additional 24 h did not dramatically change the results. [³H]CB prepared by the method of Lin, Santi, and Spudich (8) was obtained from New England Nuclear, Boston, Mass.

RESULTS

It has long been known (15) that fragments of F-actin can increase the rate of assembly of actin by providing more filament ends onto which monomers can add. It can be seen in Fig. 1 (compare open vs. filled symbols at zero cytochalasin) that fragments of F-actin accelerate the rate of assembly nearly fivefold under the conditions of the experiment. We found that cytochalasin has a dramatic effect on this fragment-accelerated rate. The concentrations required for half-maximal inhibition are 2 × 10⁻⁷ M CB and 10⁻⁸ M CD (Fig. 1).

Cytochalasin also inhibits the rate of actin assembly under these conditions without fragments added, but to a lesser extent (Fig. 1, filled symbols). However, higher than micromolar concentrations of CB have the opposite effect; for example, 5 × 10⁻⁶ M CB increases the assembly rate to 110% of the control value (Fig. 1 A, filled circles). At these higher concentrations, we also noted a dramatic increase in the rate of ATP hydrolysis by F-actin (Fig. 1 A, squares), as previously reported by Low and Dancker (12).

The most dramatic effect of cytochalasin was observed if actin was assembled in the absence of ATP (Fig. 2), suggesting that ATP interferes with the cytochalasin inhibition.[1] In the absence of

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[1] Since G-actin can denature in the absence of ATP (16), it was first necessary to ascertain that we were using
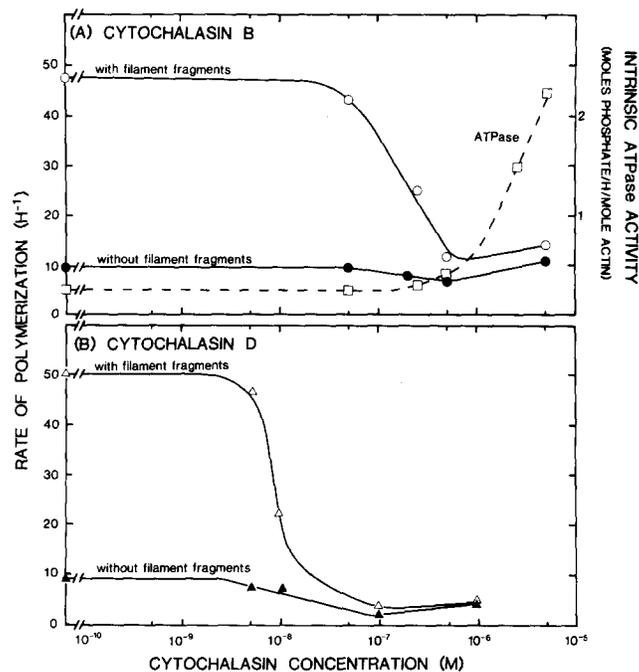
FIGURE 1 Rate of actin assembly in ATP as a function of concentration of CB (A, circles) or CD (B, triangles). Rates are expressed as the inverse of the time required to reach a reduced viscosity of 5.5 dl/g. Cytochalasin concentration is plotted on a log scale. Filled symbols represent rates of assembly at 25°C of 0.2 mg/ml Dictyostelium actin in 3 mM imidazole, pH 7.5, 0.2 mM DTT, 0.1 mM ATP upon addition of salts to 0.1 M KCl, 0.1 mM MgCl$_2$. Open symbols represent rates under these same conditions when 1 vol of 0.2 mg/ml actin filament fragments is added to 20 vol of 0.2 mg/ml G-actin simultaneously with salts. Intrinsic ATPase activity (A, squares) of 0.2 mg/ml F-actin in 3 mM imidazole, pH 7.5, 0.2 mM DTT, 0.1 mM ATP, 0.1 M KCl, 3 mM MgCl$_2$ was measured by the release of [^{32}P]orthophosphate (14).

ATP, CB has its effect in the range of 10^{-8}–10^{-7} M, and CD in the range of 5×10^{-10} to 5×10^{-9} M (Fig. 2B). These are significantly lower concentration ranges than in the presence of ATP (Table I).

The final extent of assembly under all of these conditions as measured by reduced viscosity was not greatly affected by the submicromolar concen-

conditions which adequately stabilized it. We found that G-actin remained assembly-competent in the absence of ATP for at least 24 h at 0°C if the ATP in the buffer was replaced with 3 mM ADP to saturate the nucleotide binding site (suggested by Dr. Roger Cooke, Univ. of Calif., San Francisco). As can be seen in Fig. 2A (squares), Dictyostelium actin can be assembled in this buffer to the same high reduced viscosity (~10 dl/g) as has been published for Dictyostelium actin in ATP-containing buffers (21). The assembly, however, is about an order of magnitude slower than in ATP (compare Figs. 1 and 2).

trations of cytochalasin required to lower the assembly rate (Table I). It should also be noted that CD was always about an order of magnitude more effective than CB.

The low concentrations of cytochalasin required to inhibit actin assembly suggested that high-affinity binding sites should be present in the actin preparations. Therefore, we measured binding of [^{3}H]CB to high concentrations (6–9 mg/ml) of F-actin by equilibrium dialysis. As shown by the curve in Fig. 3, Scatchard plot analysis revealed a high-affinity component with a K_d of 2×10^{-8} M, and 1 binding site/2×10^4 actin monomers (1/~60 μm of F-actin). If a straight line is fitted to all the data points in Fig. 3 by linear regression analysis, a K_d of 6×10^{-8} M and 1 binding site/~30 μm of F-actin are obtained. In the same experiments as shown in Fig. 3, the dialysis was allowed to proceed for a second day without a dramatic change in results; a line generated by
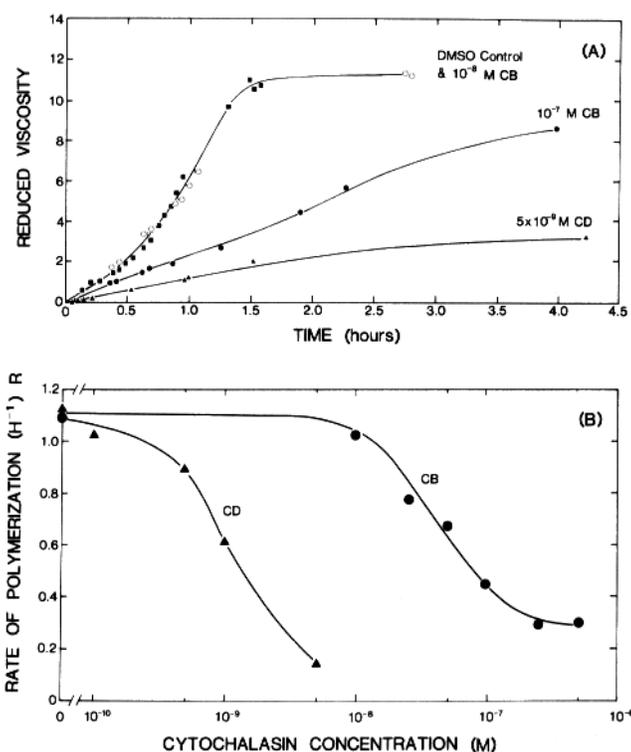
FIGURE 2 Rate of actin assembly in ADP as a function of concentration of CB (circles) or CD (triangles). Conditions are the same as in Fig. 1, except that the ATP is replaced with 3 mM ADP. (*A*) A sample of the data used to generate the dose-response curves in B. Reduced viscosity is plotted as a function of time after addition of salts (to initiate actin assembly). Actin is assembled without cytochalasin (squares), with 10^{-8} M CB (open circles), 10^{-7} M CB (filled circles) or 5×10^{-9} M CD (triangles) all in 0.5% DMSO. (*B*) Dose-response curves of the effect of cytochalasins on actin assembly in ADP.

TABLE I

Effect of Cytochalasin on Rate and Extent of Actin Assembly

Condition		Cytochalasin concentration for half-maximal inhibition	Reduction in final extent of assembly
		M	%
CB	with ATP	2×10^{-7}	6
	with ADP	4×10^{-8}	0
CD	with ATP	2×10^{-8}	20
	with ADP	1×10^{-9}	11

The concentration of cytochalasin required for half-maximal inhibition of the rate of actin assembly is taken from the data of Fig. 1 (without filament fragments) and Fig. 2 B. The final extent of assembly is measured by reduced viscosity.

linear regression analysis of these data yields a K_d of 7×10^{-8} M and 1 binding site/~20 μm of F-actin.

DISCUSSION

We report here that submicromolar concentrations of cytochalasin inhibit the rate of assembly of purified actin, under conditions where the final extent of assembly is minimally affected. The effect on assembly satisfies the criteria set up in the Introduction: it is a high affinity effect, seen at very low cytochalasin concentrations, and the proper order of effectiveness, CD > CB, is seen. Furthermore, we have demonstrated a high-affinity binding of cytochalasin to purified F-actin which corresponds to and can account for the effects on actin assembly.

Our observations are clearly distinct from a variety of lower affinity effects previously reported for purified actin. In fact, at higher cytochalasin concentrations, the opposite effect is seen: cytochalasin accelerates the rate of actin assembly (see Fig. 1 and reference 12). Other effects seen at higher cytochalasin concentrations include a re-
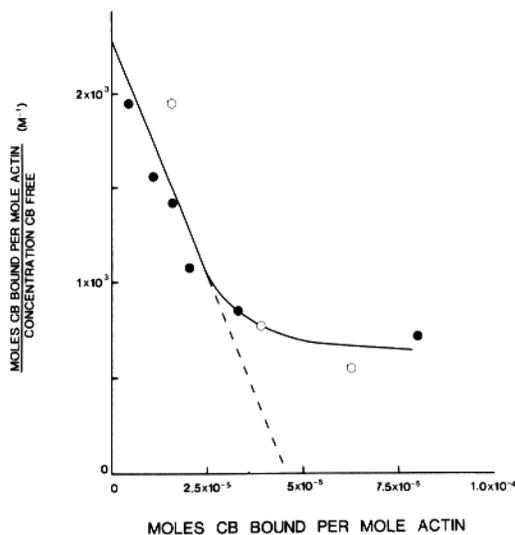
FIGURE 3 Scatchard plot analysis of the binding of [³H]CB to F-actin in ADP, as described in detail in Materials and Methods. The open and filled circles refer to different experiments.

duction in the viscosity of F-actin (18), an altered morphology of F-actin (17), and an elevated intrinsic ATPase activity (see Fig. 1 A and reference 12). Low and Dancker (12) postulate that these cytochalasin effects are due to destabilization of the actin filament and thus give results similar to those obtained with sonication of F-actin. Sonication causes fragmentation of F-actin and generates filament ends; this is at least partially responsible for the increased intrinsic ATPase and can cause increased rates of assembly (15), as for instance in our experiments with added filament fragments (Fig. 1).

Since actin appears to assemble by sequential addition of monomers to a growing filament end (3, 16), it is possible that cytochalasin inhibits the rate of assembly by interacting with an end of a filament and interfering with further addition of G-actin monomers. The stoichiometry of high-affinity binding from the Scatchard plot is ~1 site/ 20 to 60 μm of F-actin. Electron microscopy of Dictyostelium F-actin with a reduced viscosity of ~11 dl/g reveals filaments generally longer than 10 μm (21), but precise lengths are difficult to measure. Qualitatively, they are long enough to suggest that there may be on the order of one cytochalasin binding site per filament. Since this stoichiometry represents 1 binding site/~10⁴ monomers, it is, of course, possible that a very minor

non-actin component in our highly purified actin preparation is the binding species. This minor component would, nonetheless, have to be involved in actin assembly, and the simplest hypothesis is that an actin filament end is the binding species.

Another indication that cytochalasin inhibits the rate of assembly by interacting with filament ends is the observation that fragment-induced assembly is even more sensitive to cytochalasin, even though the concentration of Dictyostelium actin in those experiments was the same as in the uninduced case. Thus, the only variable was the number of filament ends, and possibly the species of nucleotide at the filament end. During assembly the ATP bound to G-actin is hydrolyzed to ADP, which remains tightly bound to F-actin (19). Since the hydrolysis step may occur subsequent to the assembly step as monomers add on to the growing filament (reference 3; Pardee and Spudich, unpublished observations), one might expect to have bound ATP at the assembling end of F-actin. Sonication, on the other hand, would be expected to generate ends having bound ADP. This thinking is illustrated in the following scheme:
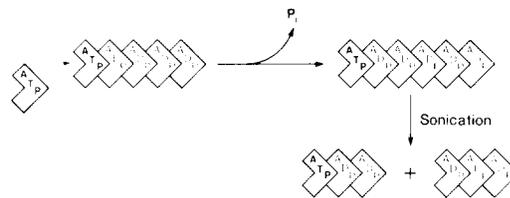
The data presented in this report indicate that ATP interferes with the cytochalasin inhibition, since assembly in the absence of ATP is so much more sensitive to cytochalasin. An interpretation consistent with both this and the filament fragment data is that a filament with ADP bound may be more susceptible to cytochalasin inhibition than an end with ATP bound.

Lin and Lin (6) have isolated a 30S complex from erythrocyte membranes which both binds cytochalasin with high affinity and induces actin assembly that is sensitive to cytochalasin. This complex contains actin, spectrin, and other minor components. Since the experiments reported here show that filament fragments dramatically increase the rate of actin assembly in a manner that is quite sensitive to cytochalasin, it is possible that the actin in their complex is in a form somewhat equivalent to the filament fragments.

Further studies on the interaction of cytochala-

sin with purified actin could lead to an understanding of cell biological effects of cytochalasin in terms of how actin assembly and disassembly might be involved in motile processes. Furthermore, insight into the role of nucleotide in regulation of assembly may be gained if cytochalasin proves to be a sensitive probe for the species of nucleotide at the actin filament end.

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