CYTOCHALASINS INHIBIT NUCLEI-INDUCED ACTIN POLYMERIZATION BY BLOCKING FILAMENT ELONGATION

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ABSTRACT

Polylysine was found to induce polymerization of muscle actin in a low ionic strength buffer containing 0.4 mM $MgCl_2$. The rate of induced polymerization was dependent on the amount and on the molecular size of the polylysine added. A similar effect was obtained by adding actin nuclei (containing about 2–4 actin subunits) cross-linked by *p-N,N'*-phenylenebismaleimide to G-actin under the same conditions, suggesting that the effect of polylysine is due to promotion of the formation of actin nuclei. Polymerization induced by polylysine and by cross-linked actin nuclei was inhibited by low concentrations (10^{-8}–10^{-6} M) of cytochalasins. Binding experiments showed that actin filaments, but not actin monomers, contained high-affinity binding sites for [^{3}H]cytochalasin B (one site per 600 actin monomers). The relative affinity of several cytochalasins for these sites (determined by competitive displacement of [^{3}H]dihydrocytochalasin B) was: cytochalasin D > cytochalasin E ≅ dihydrocytochalasin B. The results of this study suggest that cytochalasins inhibit nuclei-induced actin polymerization by binding to highly specific sites at the point of monomer addition, i.e., the elongation site, in actin nuclei and filaments.

KEY WORDS actin filaments · actin polymerization · cytochalasins · cell motility · cytoskeleton

The polymerization of globular actin (G-actin) to form filamentous actin (F-actin) in vitro can be initiated by addition of salts, e.g., 0.1 M KCl or 2 mM $MgCl_2$, to the medium. This reaction is thought to involve two distinct steps: the slow formation of nuclei consisting of three to four monomers followed by the rapid addition of monomers to the nuclei (elongation) (8).

We have recently shown that high molecular weight complexes containing actin, spectrin, and band 4.1 can be isolated from human red cell membranes (12). The addition of these complexes to G-actin in a low ionic strength medium containing 0.4 mM $MgCl_2$ causes rapid polymerization of the actin. This phenomenon is inhibited by low concentrations of cytochalasins. The relative potency of the drugs in inhibiting this type of actin polymerization corresponds to their relative affinity for binding sites in the complexes (11) and to their relative effectiveness in blocking various forms of motile functions in animal cells, i.e., cytochalasin E > cytochalasin D > dihydrocytochalasin B (1, 3, 14). Although the molecular mechanism of the complex-induced polymerization remains to be determined, it is a distinct possibility that the role of the complexes in the reaction is to provide sites, i.e., nuclei, onto which actin monomers can be added to form filaments.

Recent reports have shown that positively charged substances such as polyamines (19), H1 histone (18), protamine (18), and polylysine (2) can accelerate the rate of conversion of G-actin to

F-actin, presumably by enhancing the formation of actin nuclei. We were prompted by these results to examine the possibility that the action of cytochalasin-binding complexes in inducing actin polymerization in low ionic strength medium can be mimicked by one of these substances. We report here that polylysine and chemically cross-linked actin nuclei can induce actin polymerization under these conditions. Moreover, both types of polymerization are sensitive to low concentrations of cytochalasins and are accompanied by the formation of high-affinity binding sites for the cytochalasins. These results suggest that the site of action of cytochalasins in inhibiting nuclei-induced actin polymerization is at the growing end of actin nuclei and filaments. A preliminary report of this work has appeared in abstract form (5).

MATERIALS AND METHODS

Cytochalasins B, D, and E were purchased from Aldrich Chemical Co., Milwaukee, Wis. [^{3}H]Cytochalasin B, prepared by the method of Lin et al. (15), was obtained from New England Nuclear, Boston, Mass. Tritium-labeled and unlabeled dihydrocytochalasin B were prepared from the corresponding forms of cytochalasin B as previously described (11, 13). Polylysine preparations of various molecular sizes were obtained from Sigma Chemical Co., St. Louis, Mo. Actin was isolated from rabbit skeletal muscle by the method of Spudich and Watt (21). Cross-linked actin nuclei were prepared by reaction of F-actin with *p-N,N'*-phenylenebismaleimide for 30 minutes at 25°C according to the method of Knight and Offer (10) followed by purification on a Sephacryl S 300 Superfine (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.) column in 5 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, and 0.01% NaN_3, pH 8.0. Material from the center of the first peak, which eluted after the void volume, was pooled, concentrated by ultrafiltration (PTGC 11K immersible CX unit, Millipore Corp., Bedford, Mass.) to 0.72 mg/ml, and designated "cross-linked actin nuclei." This preparation contained about two-thirds actin dimers and about one-third monomers, as judged by SDS polyacrylamide gel electrophoresis.

Viscosity was measured at 25°C with Ostwald-type viscometers. Specific viscosity (η_{sp}) is defined as the flow time of the sample divided by the flow time of the appropriate buffer, minus 1. Cytochalasin binding was measured with the isoelectric precipitation assay as previously described (12). Protein content was determined with the Hartree (6) modification of the Lowry method.

RESULTS

Induction of Actin Polymerization

Fig. 1 shows that the addition of small amounts of polylysine (mol wt ~87,000) to a solution of G-actin in a low ionic strength medium containing 0.4 mM $MgCl_2$ resulted in rapid polymerization of the actin as measured by viscometry. In contrast, almost no increase in viscosity was observed in the control sample to which no polylysine had been added. The rate of polymerization of the actin was dependent on the amount of polylysine added to the medium. At a ratio of 1 mol of polylysine per 1,000 mol of G-actin, actin was fully polymerized in less than 1 h, i.e., the viscosity of the solution was about the same as that of a solution of F-actin formed in the presence of 0.1 M KCl or 2 mM $MgCl_2$. At the highest level of polylysine, the final viscosity of the solution exceeded that of an equivalent solution of F-actin, presumably as a result of cross-linking of the actin filaments by the polylysine (17). The induction of polymerization was also dependent on the low concentration of $MgCl_2$ in the medium; the addition of polylysine in the absence of 0.4 mM $MgCl_2$ resulted in only a very small increase in the viscosity of the actin solution. Preincubation of G-actin with polylysine followed by addition of 0.4 mM $MgCl_2$ resulted in a rate of actin polymerization at least twice that obtained when the preincubation period was omitted. These results suggest that polylysine enhanced the rate of actin nucleus formation, even in the absence of $MgCl_2$, but that filament elongation requires the presence of 0.4 mM $MgCl_2$.

As shown in Fig. 2, polylysine-induced polymerization of actin was inhibited by substoichiometric levels of cytochalasins. Cytochalasin D was the most potent inhibitor, effective in the 10^{-8}–10^{-7} M range; cytochalasin E was effective in the

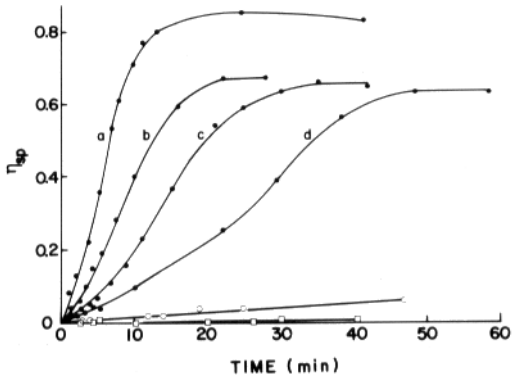

FIGURE 1 Induction of actin polymerization by polylysine. At zero time, 10 μg (*a*), 4 μg (*b*), 2 μg (*c*), or 1 μg (*d*) of polylysine (Type I-B, mol wt of 87,000, from Sigma Chemical Co.) was added to 0.5 mg of G-actin in 0.5 ml of 0.2 mM ATP/0.2 mM $CaCl_2$/0.4 mM $MgCl_2$/0.5 mM β-mercaptoethanol/5 mM Tris-HCl, pH 8.0. Polymerization of actin was monitored by viscometry. The control samples were: (□), no polylysine was added; (○), 40 μg of polylysine was added, but 0.4 mM $MgCl_2$ was omitted from the buffer.

10^{-7}–10^{-6} M range; and dihydrocytochalasin B was the least potent, effective at 10^{-6} M. These results are somewhat different from those obtained with actin polymerization induced by cytochalasin-binding complexes from human red cells. In that system, cytochalasin E was found to be a more potent inhibitor than cytochalasin D (12).

One likely explanation for the effect of polylysine on actin polymerization is that the polypeptides are positively charged surfaces that can bind several molecules of negatively charged actin monomers, thereby promoting their association to form nuclei in the polymerization reaction. According to this model, polylysine chains that are too short (or barely long enough) to span the diameters of several actin molecules should be less effective than long chains as sites of nucleation. Results in support of this idea are presented in Fig. 3. Short polylysine chains, on the basis of either mass or number of moles, were far less effective than long chains in inducing actin polymerization. The shortest chains used in this experiment (mol wt ~3,000), even if fully extended, have a length of about 94 Å, which is barely long enough to cover the sum of the diameters of two actin monomers (diameter of actin, ~50 Å).

If polylysine induces actin polymerization in low ionic strength medium by enhancing formation of actin nuclei, the addition of preformed

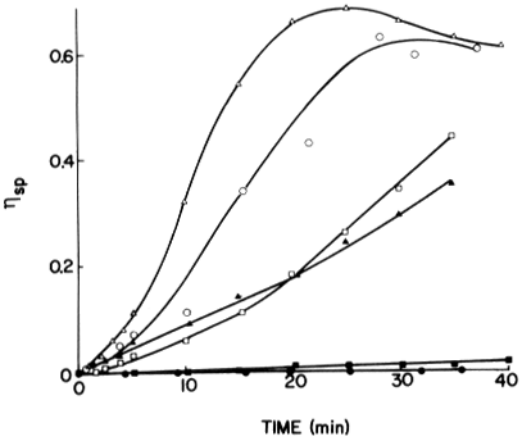

FIGURE 2 Inhibition of polylysine-induced actin polymerization by cytochalasins. At zero time, 5 μg of polylysine (Type I-B) was added to 0.5 mg of G-actin in 0.5 ml of the buffer described in Fig. 1, in the absence (△) or presence of 0.2 μM cytochalasin E (○), 2 μM cytochalasin E (●), 0.02 μM cytochalasin D (□), 0.2 μM cytochalasin D (■), or 2 μM dihydrocytochalasin B (▲). Polymerization was monitored by viscometry.

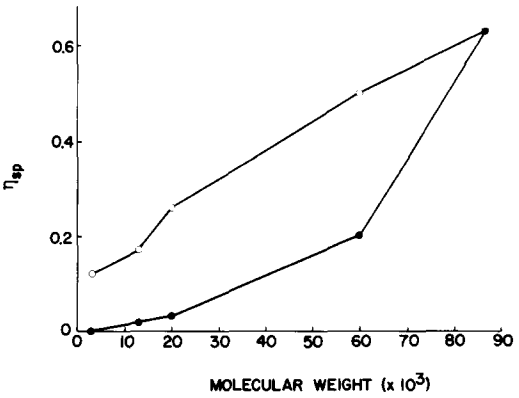

FIGURE 3 Induction of actin polymerization by polylysine of different molecular weights. At zero time, polylysine preparations with mol wt of 87,000, 60,000, 20,000, 13,000, or 3,000 were added to 0.5 mg of G-actin in 0.5 ml of the buffer described in Fig. 1. The final concentration of polylysine used in each sample was 5 μg/ml (○) or 0.05 μM (●). Viscosity was measured after 40 min at 25°C.

actin nuclei should produce the same result. We found that the addition of small quantities of actin nuclei cross-linked by the bivalent sulfhydryl reagent *p-N,N'*-phenylenebismaleimide to a solution of G-actin in 0.4 mM $MgCl_2$ did indeed lead to rapid polymerization of the actin. Addition of an equivalent amount of G-actin did not produce this effect (Fig. 4). As was the case with polylysine, the rate of polymerization was dependent on the number of cross-linked actin nuclei[1] added, and the reaction was sensitive to low concentrations of cytochalasins (Fig. 5).

High-affinity Cytochalasin Binding Sites Associated with Actin

The sensitivity of actin polymerization induced by polylysine and by cross-linked actin to low concentrations of cytochalasins indicates that high-affinity binding sites for cytochalasin must be present in these systems. To test this hypothesis, we measured the binding of [^{3}H]cytochalasin B to the various forms of actin used in the polymeri-

[1] Although electrophoresis in SDS polyacrylamide gels showed that this preparation contained predominantly actin monomers and dimers, it is possible that the active species were trimers or tetramers formed by the association of these two components. However, since the preparation consisted of material eluted after the void volume in a Sephacryl S 300 column, the actin should not be in a polymer of greater than four or five subunits.

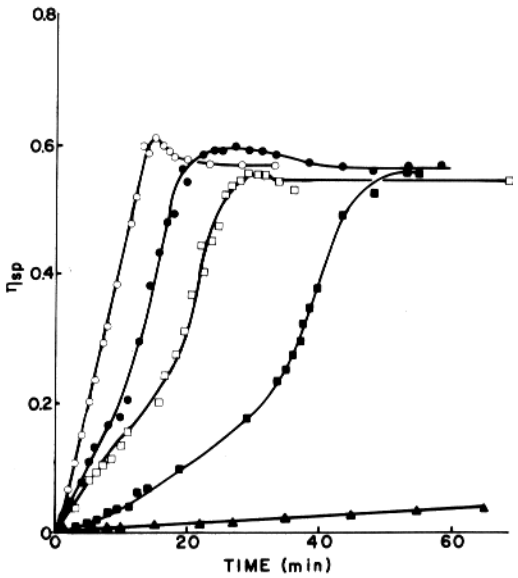

FIGURE 4 Induction of actin polymerization by cross-linked actin nuclei. G-actin (0.5 mg) was incubated in the absence (▲) or presence of 44 μg (○), 22 μg (●), 11 μg (□), or 5.5 μg (■) of cross-linked actin nuclei in 0.5 ml of the buffer described in Fig. 1 for 10 min at 25°C. At zero time, 0.4 mM $MgCl_2$ was added to the samples and polymerization of the actin was monitored by viscometry.

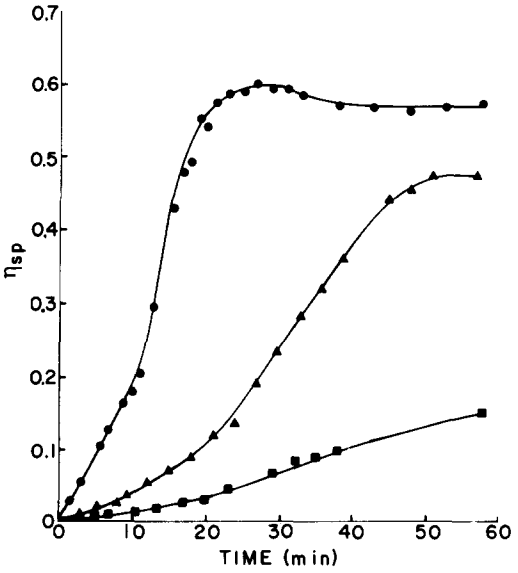

FIGURE 5 Inhibition of nuclei-induced actin polymerization by cytochalasins. G-actin (0.5 mg) was incubated with 22 μg of cross-linked actin nuclei in 0.5 ml of the buffer described in Fig. 1, which contained no cytochalasin (●), 0.6 μM cytochalasin B (▲), or 0.1 μM cytochalasin D (■), for 10 min at 25°C. At zero time, 0.4 mM $MgCl_2$ was added to the samples and actin polymerization was monitored by viscometry.

zation studies described above by using the isoelectric precipitation assay. The following results were obtained by Scatchard plot analysis of the binding data (Fig. 6): Firstly, G-actin, by itself, had no measurable high-affinity binding sites (data not shown).[2] Secondly, cross-linked actin nuclei also had no high-affinity binding sites (data not shown).[2] However, subsequent experiments showed that high-affinity cytochalasin binding complexes from red cells lost most of their binding activity after reaction with *p-N,N'*-phenylenebismaleimide, indicating that the cross-linking reagent can destroy cytochalasin binding activity. Thirdly, a solution of G-actin and polylysine in the absence of $MgCl_2$ had about 1 mol of high-affinity binding site (dissociation constant ~4 × 10^{-9} M at pH 5) per 3,000 mol of actin. Finally, a solution of F-actin formed in the presence of polylysine and 0.4 mM $MgCl_2$ had about 1 mol of high-affinity binding site (dissociation constant ~6

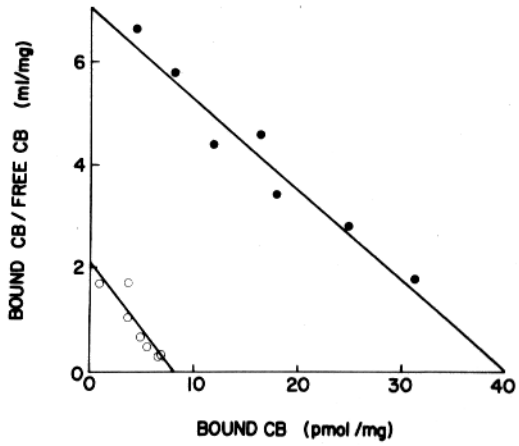

FIGURE 6 Scatchard plot analysis of the binding of [^{3}H]cytochalasin B to actin incubated with polylysine. G-actin (1 mg) was incubated with 10 μg of polylysine (Type I-B) in 1 ml of 0.2 mM ATP/0.2 mM $CaCl_2$/0.5 mM β-mercaptoethanol/5 mM Tris-HCl, pH 8.0, with (●) or without (○) 0.4 mM $MgCl_2$. After 40 min, 50-μl aliquots were assayed for cytochalasin-binding activity in 0.5 ml of buffer with the use of the isoelectric precipitation method. The concentration range of [^{3}H]cytochalasin B used in this experiment was 2–40 nM.

[2] Binding was measured in buffer that contained 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 5 mM Tris-HCl, pH 8.0.

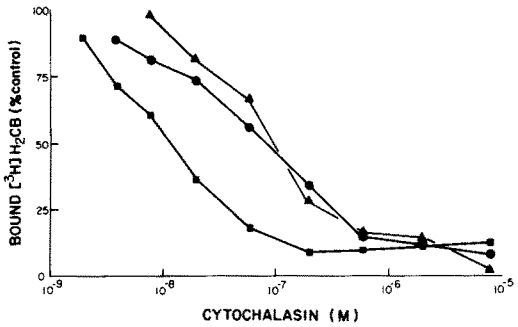

FIGURE 7 Displacement of [^{3}H]dihydrocytochalasin B bound to F-actin by unlabeled cytochalasins. Binding of [^{3}H]dihydrocytochalasin B (12 nM) to F-actin (85 μg per 0.5 ml) polymerized in 2 mM $MgCl_2$ was measured in 0.2 mM ATP/0.2 mM $CaCl_2$/2 mM $MgCl_2$/0.5 mM β-mercaptoethanol/5 mM Tris HCl, pH 8.0, containing various concentrations of unlabeled cytochalasin D (■), cytochalasin E (▲), or dihydrocytochalasin B (●). Binding is expressed as percent of a control sample to which no unlabeled cytochalasin was added (100% = 2.4 pmol [^{3}H]dihydrocytochalasin B bound per sample).

$\times 10^{-9}$ M at pH 5)[3] per 600 mol of actin. Similar results were obtained with F-actin formed in the presence of cross-linked actin nuclei in 0.4 mM $MgCl_2$ or F-actin formed in 2 mM $MgCl_2$ (data not shown). These results indicate that high-affinity cytochalasin B binding sites are associated with actin only when the protein is in filamentous form, i.e., long filaments or short nuclei, and not when it is in monomeric form.

To correlate cytochalasin binding with inhibition of actin polymerization, we studied the relative affinity of several cytochalasins for the binding sites associated with F-actin by using a competitive displacement assay (11). As shown in Fig. 7, the relative effectiveness of the unlabeled cytochalasins in displacing [^{3}H]dihydrocytochalasin B from F-actin was: cytochalasin D > cytochalasin E ≅ dihydrocytochalasin B. This is in general agreement with the results of the viscosity experiment in which we found that the relative potency of the drugs in inhibiting polylysine-induced polymerization was: cytochalasin D > cytochalasin E > dihydrocytochalasin B. Why the apparent affinity of cytochalasin E for sites associated with F-actin was not significantly greater than that of dihydrocytochalasin B (as might be expected on the basis of the polymerization experiment) remains to be determined.

[3] The dissociation constant for binding of [^{3}H]cytochalasin B to F-actin measured with an equilibrium dialysis method at pH 8 (11) is 5×10^{-8} M.

DISCUSSION

We have previously shown that cytochalasin-binding complexes purified from human red cell membrane (12), bovine brain (4), and human platelet (16) greatly accelerated actin polymerization in low ionic strength buffer containing 0.4 mM $MgCl_2$. We show here that the addition of small amounts of polylysine to G-actin under the same conditions also results in rapid polymerization of the actin. The role of polylysine in this experiment may be that of a "binding agent" that increases the rate of nucleus formation by promoting the association of negatively charged actin monomers. This model is consistent with our finding that actin nuclei formed by cross-linking actin molecules with a sulfhydryl reagent can also induce actin polymerization in the same manner as polylysine. The results presented here are in support of our view that cytochalasin-binding complexes act as nuclei in the induction of actin polymerization in low ionic strength medium (12, 16).

Previous work performed in this laboratory has shown that actin polymerization induced by cytochalasin-binding complexes (4, 12, 16) or muscle F-actin (5) in low ionic strength medium are inhibited by low concentrations (10^{-8}–10^{-6} M) of cytochalasins. The data presented here demonstrate that actin polymerization induced by polylysine and by cross-linked actin nuclei are also affected by the drugs under similar conditions. All of these cytochalasin-sensitive polymerization reactions share a common mechanism: polymerization is either induced or accelerated by some form of nucleus onto which actin monomers can be added. Therefore, the action of the cytochalasins in all of these reactions is likely to be directed at the point of filament elongation located at the ends of the actin nuclei or filaments.

The binding experiments with radioactively labeled cytochalasins support the proposal that the drugs interact with highly specific sites located in actin filaments and nuclei. We found that G-actin did not bind cytochalasin B with high affinity. However, conversion of G-actin to F-actin resulted in formation of a significant number of high-affinity [^{3}H]cytochalasin B binding sites. The number of sites detected (approximately one site per 600 actin monomers) is on the same order as the number of actin molecules estimated to be in an

average actin filament under similar conditions (9). The calculated dissociation constant for the binding reaction measured at pH 8 (5×10^{-8} M) is lower than the effective concentration of cytochalasin B in inhibiting actin polymerization. This difference can be explained by the fact that the actin concentration used in the polymerization experiments (1 mg/ml) was much higher than that used in the binding assays (0.1 mg/ml). The experiments on the inhibition of nuclei-induced actin polymerization by cytochalasin discussed above suggest that these sites are located at the growing ends of the actin nuclei and filaments. The finding that a mixture of G-actin and polylysine in the absence of 0.4 mM $MgCl_2$ contained high-affinity sites could be explained by the formation of actin nuclei that were too short to be detected by viscometry.

The differential potencies of several cytochalasins have been used to relate high-affinity cytochalasin-binding structures to the cytoskeletal-contractile functions of the cell. The relative effectiveness of cytochalasins in inhibiting actin polymerization induced by cytochalasin-binding complexes has been determined to be: cytochalasin E > cytochalasin D > dihydrocytochalasin B. This order corresponds to the relative potency of the drugs in inhibiting many types of motile functions in animal cells (1, 3, 14). The results presented here show that the relative effectiveness of cytochalasins in inhibiting actin polymerization induced by polylysine is: cytochalasin D > cytochalasin E > dihydrocytochalasin B. This difference in differential sensitivity to cytochalasins suggests that other proteins in the cytochalasin-binding complexes possibly exert an effect on the substrate specificity of the high-affinity cytochalasin-binding sites associated with the actin in the complexes. Further investigation is required to clarify this point.

The structure of actin filaments in vitro has been found to be disrupted by stoichiometric levels of cytochalasins (20). The relative effectiveness of the cytochalasins in producing this effect corresponds to the relative potency of the drugs in inhibiting cell motility (7). The results presented here show that substoichiometric levels of cytochalasins can block nuclei-induced formation of actin filaments. These two effects could be the basis for many of the effects of cytochalasins on cell motility and cell morphology reported in the literature.

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