Cytochalasin B Slows But Does Not Prevent Monomer Addition at the Barbed End of the Actin Filament

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Abstract. We used *Limulus* sperm acrosomal actin bundles to examine the effect of 2 μM cytochalasin B (CB) on elongation from both the barbed and pointed ends of the actin filament. In this paper we report that 2 μM CB does not prevent monomer addition onto the barbed ends of the acrosomal actin filaments. Barbed end assembly occurred over a range of actin monomer concentrations (0.2–6 μM) in solutions containing 75 mM KCl, 5 mM $MgCl_2$, 10 mM Imidazole, pH 7.2, and 2 μM CB. However, the elongation rates were reduced such that the rates at the barbed end were approximately the same as those at the pointed end. The association and dissociation rate constants were 8- to 10-fold smaller at the barbed end in the presence of CB along with an accompanying twofold increase in critical concentration at that end. Over the time course of experimentation there was little evidence for potentiation by CB of the nucleation step of assembly. CB did not sever actin filaments; instead its presence increased the susceptibility of actin filaments to breakage from the gentle shear forces incurred during sample preparation. Under these experimental conditions, the assembly rate constants and critical concentration at the pointed end were the same in both the presence and the absence of CB.

CYTOCHALASINS have long been used as probes for studying actin-based motility and cytoskeletal structure (43, 51, 52). Since these agents affect actin-mediated events in vivo, considerable effort has been devoted to understanding the actin-cytochalasin interaction in vitro (see references 21, 23, and 25 for reviews). It is generally agreed that the cytochalasins slow the rate of filament polymerization by inhibiting the rate of elongation (5, 7, 8, 17, 22, 24, 28, 30, 39, 50). This action is produced by the high-affinity binding of the cytochalasin to the barbed, fast growing, end of an actin filament, preventing monomer addition, so called "capping" (8, 17, 28, 33, 39). Whereas a number of experiments support this conclusion, the most compelling evidence for such a capping mechanism are the nucleated actin assembly studies, which used either S_1-decorated filament fragments or microvillar actin bundles, in which cytochalasin B (CB)[1] prevented barbed end filament growth from these nuclei (28, 33, 38). Whereas the primary site of action for the cytochalasins reside at the barbed filament end, these drugs can have additional interactions with actin. For example, it has been shown that CB alters the steady state length of actin filaments (30, 42). It has been postulated that such length-regulating activity could be due to the ability of cytochalasin to nucleate filament assembly or prevent filament annealing, or to increase the filaments' susceptibility to spontaneous breakage or even induce filament severing (14, 21, 30, 42). There is even suggestive evidence that CB might decrease the rate of elongation occurring from the pointed end of the filament (39, 48). Such an effect could be due to another, as yet uncharacterized, cytochalasin-actin filament interaction or to a cytochalasin-actin monomer interaction (6). Furthermore, the activity of cytochalasin may be dependent on the conditions of experimentation (e.g., shear effects, ionic conditions), making it difficult to define a general mechanism of the interaction of cytochalasin with actin (see for example reference 39).

To examine quantitatively the effects of CB on the assembly of actin at the two ends of a filament, we used the *Limulus* acrosomal bundles as a nucleus for actin assembly using previously established methods (2, 4, 10–13, 26, 36, 39). We determined the effects of CB on the elongation rate constants and critical concentrations (C_c's), for both ends of the actin filament in the presence of KCl and Mg^{++}. Surprisingly, it was observed that 2 μM CB does not "cap" the barbed end of the filament, although it does slow the rate of elongation. CB has also a slight effect on the C_c at the barbed end. These experiments also allow the examination of CB effects on

[1] *Abbreviations used in this paper:* CB, cytochalasin B; C_c, critical concentration; DMSO, dimethyl sulfoxide.

Table I. Summary of C_o's and Elongation Rates in 2 μM CB

	Elongation rates		
	4 μM	6 μM	C_o
	s^{-1}	s^{-1}	μM
Barbed end	4.3 ± 0.02	7.3 ± 0.5	0.2
Pointed end	3.7 ± 0.3	5.9 ± 0.8	0.6

Conditions: 75 mM KCl, 5 mM $MgSO_4$, and 10 mM imidazole (pH 7.2).

nucleation and filament stability.

A preliminary report of this work was presented at the 23rd Annual Meeting of the American Society for Cell Biology (3).

Materials and Methods

Materials

Limulus polyphemus were obtained from the Marine Biological Laboratory, Woods Hole, MA. Sperm were collected from ripe males by either manual or electrical stimulation. CB, cytochalasin D, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of Limulus Acrosomal Process Bundles and Actin

Limulus sperm acrosomal actin bundles were isolated according to the methods of Tilney (46) with a slight modification as described elsewhere (2). Once isolated the bundles remained usable for several days when stored as a pellet on ice. For extended storage the isolated processes can be kept at −20°C in buffers containing 50% glycerol. For the experiments reported here freshly prepared acrosomal bundles were used, whereas the glycerol-stored bundles were used only for preliminary experiments. Actin was prepared from acetone powders of chicken breast muscle according to Spudich and Watt (41) and stored at 8 mg/ml while continually dialyzing against 2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.2 mM ATP, and 0.02% NaN_2 (the dialysis buffer was changed every 24–48 h). Actin prepared this way retains its ability to polymerize for several weeks. All the reported experiments used actin within 7–10 d after extraction from the acetone powder. Pure actin monomer was obtained by gel filtration on Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) (27) and those column fractions either at or immediately after the monomer peak were used for the experiments. Gel filtered actin was used within 24 h after elution from the column. Actin concentrations were determined spectrophotometrically using $A^{1\%}_{290} = 6.5$.

Measurement of Assembly Rate Constants and C_o

CB was dissolved in DMSO at a concentration of either 2 or 20 mM and stored at −20°C. The CB was added to *Limulus* acrosomal bundles and incubated under assembly salt conditions (see below) at 22°C for 15–30 min. After this incubation an aliquot of gel-filtered G-actin was added to the CB-acrosomal bundle mix, incubated for various periods, and negatively stained. Incubations ranged from 45 to 90 s at 4 μM actin and 30–90 s for 6 μM actin. The final assembly solutions contained 2 μM CB, 75 mM KCl, 5 mM $MgCl_2$, 10 mM Imidazole, pH 7.2, 20% actin storage buffer, and 0.1% DMSO. Control preparations lacked only CB. Filament lengths were measured directly from electron micrographs, either with a map reader or an electronic planimeter. The assembly rates were calculated by linear regression of length versus time plots. Rate constants were obtained by plotting the elongation rates and C_o's versus monomer concentration and calculating the slopes and intercepts (1, 4, 36, 39, 48) by linear regression. The C_o's for each end of an actin filament, in the presence of CB, were also determined using the acrosomal bundles (4). The final assay mix contained 75 mM KCl, 5 mM $MgCl_2$, 10 mM Imidazole-Cl, pH 7.2, 10% actin storage buffer, and 0.1% DMSO. Actin concentrations ranged from 1 to 0.05 μM and incubation times ranged from 4 to 80 min depending on the actin concentration. For example, times of 4, 10, and 40 min were used for 1, 0.4, and 0.1 μM actin monomer, respectively. After incubation, the assay mixes were negatively stained and examined with an electron microscope. Micrographs of 25–50 bundles were examined for the presence or absence of nucleated filament growth. A detailed description of the above methodology and analyses are presented in an article by Bonder et al. (4).

Electron Microscopy

Negative staining was performed by applying samples to glow-discharged copper grids coated with collodion and stabilized with carbon. After 15–30 s the samples were negatively stained with 0.5–1% unbuffered aqueous uranyl acetate. Grids were examined with either a Phillips 201 or 300 at an accelerating voltage of 80 kV.

Results

Filament Elongation in the Presence of 2 μM CB

The ability of *Limulus* acrosomal bundles to nucleate actin polymerization makes it possible to quantitate the rate of filament elongation by electron microscopy (2, 4, 10, 13). Previously, it was demonstrated that all the actin filaments within the bundle are unidirectionally polarized with their barbed ends at the thinner end of this tapered actin bundle (47). The tapered morphology of the bundle makes it possible to determine, at a glance, at which end of the actin filament monomer addition is occurring (see references 2 and 4 for a detailed presentation of this assay).

Addition of gel-filtered actin monomer and salt to the *Limulus* acrosomal bundles results in the rapid polymerization of filaments from the ends of the bundles' filaments. In the absence of CB, filaments assemble faster from the barbed end (thinner bundle end) than from the pointed end (thicker bundle end). The presence of 2 μM CB abolished the strongly biased nature of filament elongation off the acrosomal actin bundles (Fig. 1 and Table I). At an actin concentration of 6 μM, the lengths of both newly assembled barbed and pointed end filaments appeared to grow to approximately the same length after a 30-s incubation (Fig. 1). This result was quite startling given the presumed capping effect of CB at the barbed end of actin filaments (8, 24, 28, 33, 39). We, therefore, repeated the experiments with three different preparations of CB, all of which yielded comparable results. In fact, these same preparations of CB capped the barbed ends of microvillar core actin filaments (33). Essentially identical results were obtained using 2 μM cytochalasin D and 2 μM actin monomer (data not shown). In control preparations without CB but with the appropriate concentrations of DMSO, actin filament elongation proceeded with the same kinetics as in samples without DMSO (also see reference 39). Thus, we will rely on the published rate constants and C_o's (4) for analysis of the effect of CB on polymerization. In fact most of the experiments presented here were conducted in tandem with our earlier studies (2, 4).

It appears that all of the filament ends of the acrosomal bundle nucleated the assembly of muscle actin filaments and that the lengths of the newly grown filaments were quite uniform. However, at later time points, when long filaments were formed breakage of filaments along their length was observed (Fig. 5). The significance of this observation is discussed in greater detail below. Short randomly nucleated filaments not associated with the actin bundles were not seen on the electron microscopy grids, indicating that the free CB was not nucleating filament assembly.

In the presence of CB, plots of average filament length versus incubation time resulted in straight line plots whose calculated slopes provide the elongation rate (Fig. 2 and Table I). The elongation rate at the barbed end was significantly lower than equivalent preparations that did not contain CB (Fig. 2). For example, in the absence of CB, barbed end

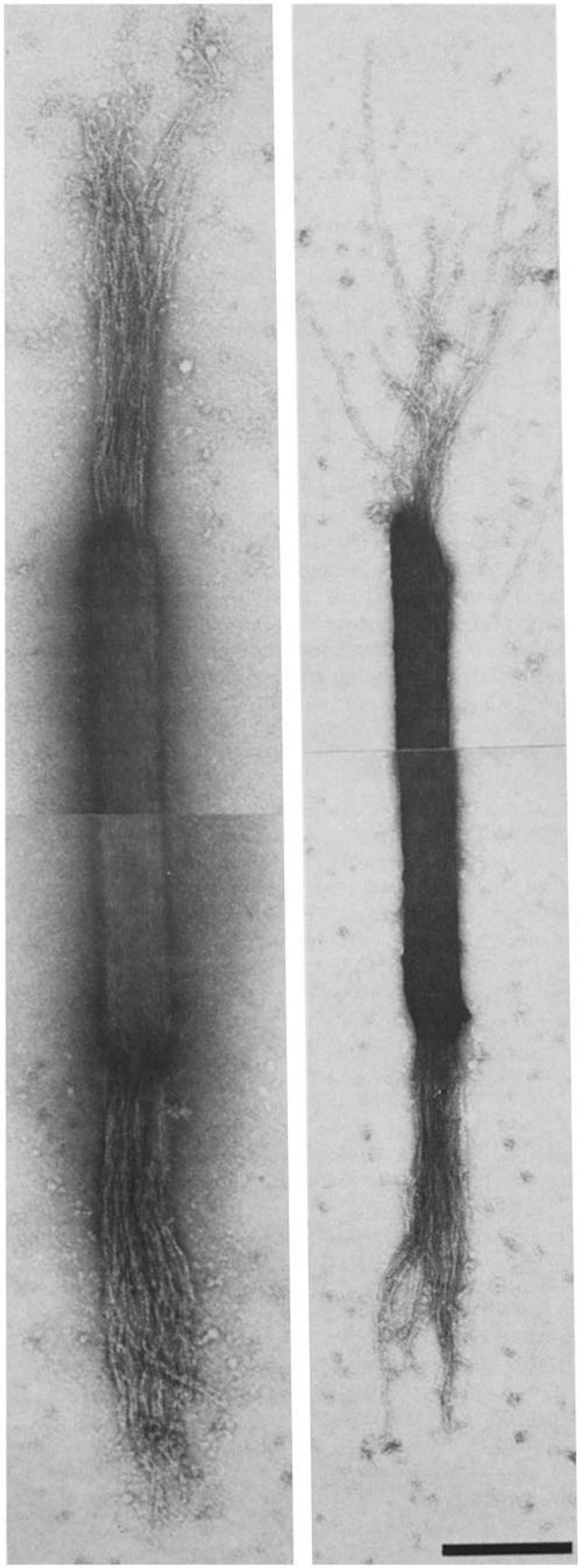

Figure 1. Nucleated assembly in the presence of CB. *Limulus* acrosomal bundles were incubated in 2 μM CB and salt, and then gel-filtered actin monomer was added to a final concentration of 6 μM. The panels are representative micrographs of filament growth off the ends of *Limulus* processes. Each panel is a composite micrograph comparing both ends of the bundles; the lower panel corresponds to pointed end assembly, the upper panel to barbed end assembly. Bar, 0.2 μm.

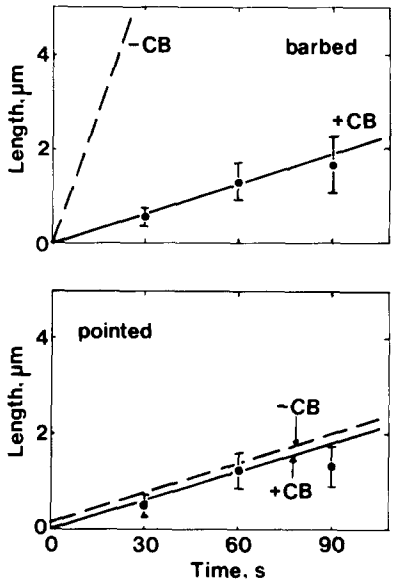

Figure 2. Elongation rates in the presence of CB. Plot of average filament length versus incubation time using 6 μM actin and 2 μM CB. Elongation rates were determined by linear regression analysis. *Top*: Rate of elongation at the barbed filament given by solid line. Over the time course of the experiment elongation was linear and the correlation coefficient was >0.99. *Bottom*: Linear elongation at the pointed filament end. Note the plateauing of the average filament length between 60 and 90 s which deflects the line downward. Correlation coefficient, >0.95. Predicted plots for elongation in the absence of CB are depicted by a dashed line (4). Errors represent 1 SD.

elongation normally occurs at a rate of ~48.5 molecules/s at 4 μM monomer, whereas the equivalent preparation in 2 μM CB elongated at ~4.6 molecules/s, a decrease of ~90%. By contrast, elongation of pointed end filaments may be decreased by 30%. This decrease may be inflated since there is a slight plateauing in the average filament length at later time points (Fig. 2), which will artifactually lower the calculated rate of elongation. If the last data points are omitted from the calculations, the elongation rates in the absence and presence of CB are virtually identical for the pointed end of the filament.

Effect of CB on the C_o for Filament Elongation

The nucleated filament assembly assay with *Limulus* acrosomal bundles can also be used to determine the minimal concentration of actin monomer required for filament elongation (4). By assaying for the presence of filaments growing from the acrosomal actin bundle ends at progressively lower concentrations of actin, one can readily identify the lowest actin concentration that can support barbed or pointed end growth. This minimal concentration is our operational definition of C_o (4). In control preparations containing KCl, Mg^{++}, and 0.1% DMSO, the barbed filament end has a lower C_o than does the pointed end (0.1 vs. 0.6 μM, respectively). These same values were also obtained in the absence of DMSO (4).

CB did not dramatically alter either the barbed or the pointed filament end C_o as compared with control preparations (compare Fig. 3, *top* with *bottom*). In one experiment, 0.3 μM monomer was needed to initiate elongation off barbed ends in the presence of 2 μM CB (Fig. 3, *bottom*). This is an actin concentration three times greater than needed to initiate assembly in the absence of CB (Fig. 3). In other experiments, we have found that barbed end elongation can be supported by as little as 0.2 μM monomer. There is always this small, but consistent, 0.1–0.2 μM increase in the barbed end C_o over control preparation. A C_o of 0.2 μM was used in the calculation of assembly rate constants (see next section). CB exerted no detectable effect on the C_o at the pointed filament end. Both controls and experimentals had C_o's of 0.6 μM (Fig. 3).

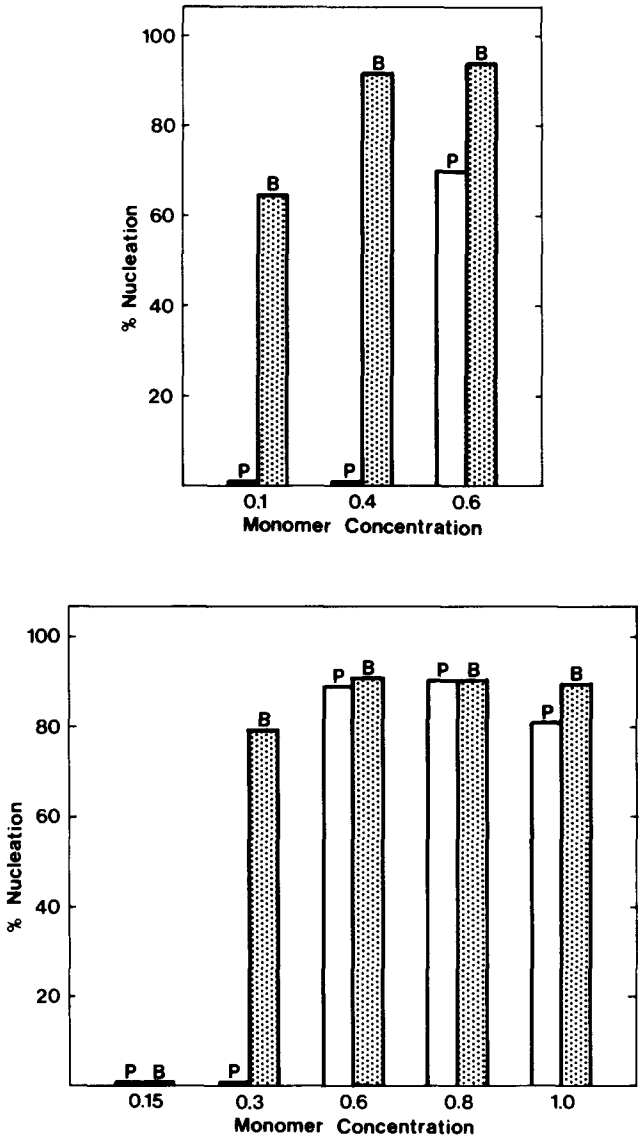

Figure 3. C_o's in the presence and absence of CB. *Top*: Control preparation in the absence of CB. Nucleated assembly was performed as described in Materials and Methods and then at least 25 bundles were examined for barbed and/or pointed filament elongation. *Bottom*: C_o in the presence of CB. Note the increase in the barbed end C_o from 0.1 μM (*top*) to 0.3 μM without any change in pointed end C_o. Stippled bars shows the percentages at the barbed end, nonstippled bars at the pointed end.

Therefore, within the limits of detection using the *Limulus* assay, CB causes a small but measurable increase in the barbed end C_o and no detectable effect on the C_o at the pointed end of the filament.

Elongation Rate Constants in the Presence of CB

Elongation rate constants were estimated by plotting the rate of elongation and C_o's as a function of actin monomer concentration. When this relationship is linear the association rate constant (k_+) is obtained from the slope; the dissociation rate constant (k_-) from the *y*-intercept; and C_o from the *x*-intercept (1, 35, 39). Recently, Pollard (37) has demonstrated that the constants measured in nucleated assembly experiments are probably for the ATP-actin monomer rather than the ADP-actin species. When the measured C_o's and the elongation rates, in the presence of 2 μM CB, were plotted the results were linear for both the barbed and pointed ends of the actin filament (Fig. 4). As might be predicted from the results (Figs. 1 and 2), the slopes for both the barbed and pointed filament ends are fairly parallel over the range of actin concentrations tested, indicating very similar k_+'s.

When the CB rate constants at the pointed end were compared with those derived in the absence of CB, there were only minor differences. The calculated rate of monomer addition is ~25% slower in the presence of CB. Whether this change is truly significant remains unclear since the deflection (plateauing) of the last time points during the elongation rate determination experiments could, as stated above, lead to an underestimation of the elongation rates. If the "weighted" rates of elongation are plotted, there appears to be no effect of CB on the association rate constant for the pointed end. The dissociation rate constant at the pointed end remains unaltered by the presence of CB.

CB dramatically reduced the rates of both monomer addition and monomer loss from the barbed end of actin filaments. There was an almost 10-fold reduction in both the k_+ and the k_- at the barbed end (Table II). This somewhat proportional reduction in rate constants helps explain how the overall elongation rate can be reduced by 90% (see above)

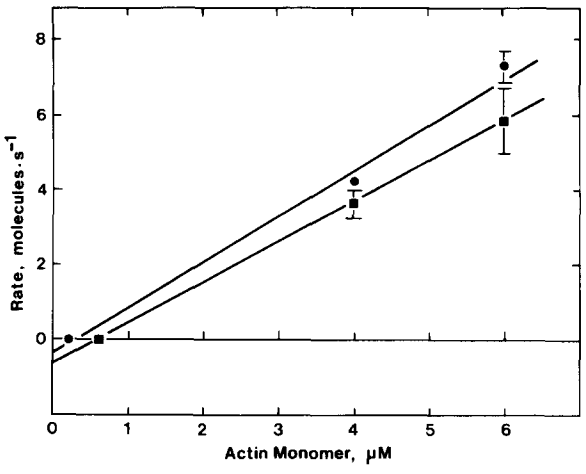

Figure 4. Estimation of elongation rate constants in presence of CB. Graphing the C_o and elongation rates versus monomer concentrate (Table I) provides linear plots for both the barbed (●) and pointed (■) filament ends. Error bars represent one standard deviation. Correlation coefficient, >0.99.

Table II. Summary of Elongation Rate Constants

	+CB	−CB*
k_+^B	1.2	12.3
k_+^P	1.1	1.5
k_-^B	0.4	2.0
k_-^P	0.6	0.7

Units; k_+, monomers per second per micromolar; k_-, monomers per second. Conditions: 75 mM KCl, 5 mM $MgSO_4$, and 10 mM imidazole (pH 7.2).
* Bonder et al. (4).

without a great change in the C_o. The CB effect on filament elongation resulted from the lowering of both the k_- and the k_+ at the barbed end of the filament, while it had no substantial effect on assembly from the pointed end.

From the rate constants we can also calculate the steady state C_o in the presence of 2 μM CB. In the presence of CB a steady state C_o of 0.4 μM is obtained, as compared with 0.2 μM in the absence of CB. CB therefore increases the steady state C_o but not to that of the pointed end, as occurs with a bona fide barbed end capping protein (2, 12, 49, 50).

Cytochalasin's Effect on Filament Stability

Over the time course of the experiments, the filaments growing off the acrosomal bundles tended to stay closely associated in a rather loose bundle. In cases where the filaments did splay from the loose bundle breaks sometimes occurred along the filament in the presence of CB (Figs. 1 and 5). This breakage was more often observed when the incubations were extended so that the filaments grew to >2 μm. Under these circumstances there was a greater tendency of the growing filaments to splay apart. Examination of the long filaments clearly demonstrate the random breaks that occurred along the filament (Fig. 5). This result cannot solely be attributed to sample handling since in the absence of CB uniform filament lengths of up to 3.5–4 μm can be attained in this assay without incurring significant breakage.

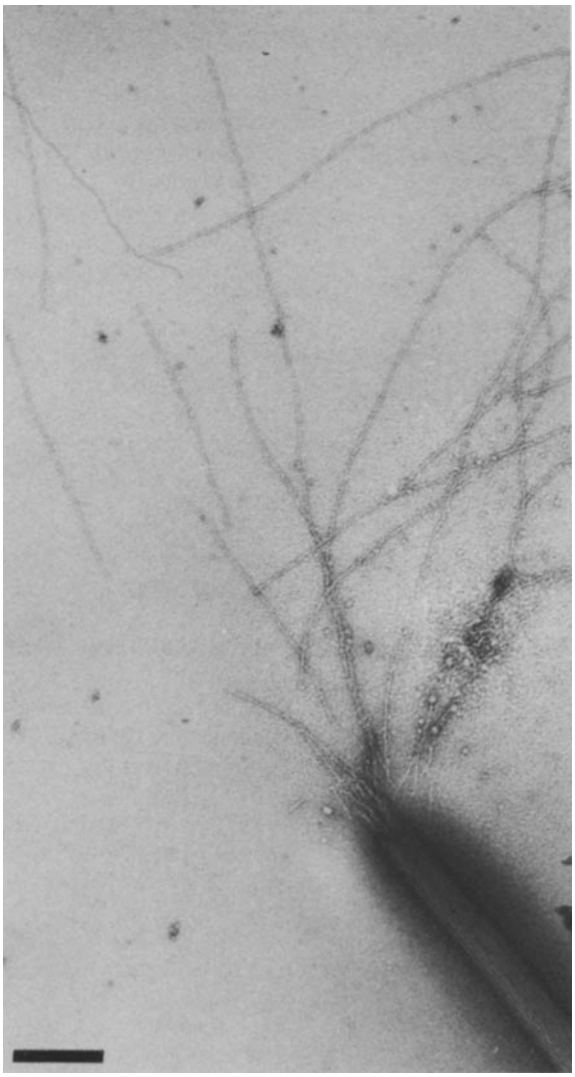

Figure 5. Filament instability in the presence of CB. Nucleated assembly using *Limulus* acrosomal bundles was performed as described in Materials and Methods with 1 μM actin monomer and 2 μM CB. The 10-min incubation used allowed for the growth of long filaments. Note the presence of breaks along the length of the newly grown filaments. Bar, 0.2 μm.

Discussion

CB Slows But Does Not Completely Inhibit Barbed End Assembly

The discovery of actin binding proteins that can regulate the assembly of actin has led to a resurgence of interest in the mechanism of actin assembly. Since the cytochalasins have been found to produce profound effects on actin polymerization (5, 7, 17, 23, 28, 33, 39, 42, 44, 50) and actin gelation (19, 28, 30, 51) they have often been used in studies of actin assembly. Currently, the dogma of the interaction of CB with actin is that it binds the barbed end of the actin filament with high affinity (K_d − ~10^{-7}–10^{-8} M), thereby stopping monomer addition (5, 8, 17, 28, 33, 39, 53). Thus, the CB effect is often equated with that of the high affinity capping proteins (21, 23).

Previously, using the *Limulus* acrosomal process nucleation assay, we demonstrated that filament elongation does not occur off the barbed ends of acrosomal actin filaments in the presence of the capping protein villin (2). This effect was specific for the barbed end since no detectable change in the elongation rates of pointed end growth off the *Limulus* acrosomal process filaments was observed (2). Therefore when villin binds the barbed end of the filament it prevents not only monomer association but also dissociation (2, 49, 50). The acrosomal process experiments were also used to demonstrate villin's ability to sever filaments and its potency at nucleating filament assembly.

Unlike with villin, elongation from the barbed end does occur in the presence of 2 μM CB, a concentration reported to completely cap the end (8, 17, 28, 33, 39). Recently, two other reports have presented evidence showing barbed end assembly in the presence of CB, although the significance of these observations has not been generally noted (26, 48). Furthermore, a concentration of 2 μM CB is well in excess of the number of barbed ends such that all barbed ends should have CB bound. In the presence of 2 μm CB, the rates of elongation from the barbed end were significantly slower than in control preparations. The growth was linear out to a filament length of at least 2 μm, at which point both the barbed and the pointed end elongation started to plateau. This slowing of elongation could be attributed to monomer

depletion resulting from the net growth of >4 μm of new filaments as well as other possible secondary CB effects (see reference 21 for review). CB decreased both the association and the dissociation rate constants by ~10-fold at the barbed end, as compared with controls (Table II). This almost proportional reduction in both rate constants is in agreement with the small (twofold) increase in C_o (Table II). In all the experiments performed, short spontaneously nucleated filaments were not seen on the electron microscopy grids in the presence of CB. Such short filaments, in this assay, are indicative of the presence of a factor that has potent nucleating activity (2). Given the absence of short filaments, it appears that 2 μM CB does not rapidly nucleate filament assembly under the present experimental conditions. This observation agrees with previous reports using similar salt conditions (28, 50). Likewise, these experiments provided little evidence for CB having significant severing activity as compared, for example, with villin using this same assay (2). What is observed is that filaments emanating from the bundle sometimes have breaks along their length. Furthermore, this breakage phenomenon occurs more frequently with longer filaments, which even in the absence of CB are naturally more susceptible to breakage during sample preparation. Therefore, since all of the newly grown filaments elongate with uniform rates in the presence of CB and only the longer filaments appear to break with a high frequency, we conclude that CB increased the susceptibility of filaments to breakage from the shear forces exerted during sample preparation.

Recently, it has been reported that the capping of one end of an actin filament results in a decrease of filament growth at the opposite filament end (31). In the experiments reported here, the CB effect is specific for the barbed end with no significant change in the assembly rate constants or C_o at the pointed end. This is in agreement with our prior experiments with villin, in which no detectable change in pointed end elongation was observed. The apparent disagreement between our data and those of Maruyama and co-workers (29, 31) is not easily explained. Although the possibility that an actin filament has the potential to relay information along its length is certainly exciting, we have not detected such an effect. Further experimentation is needed to determine why such differences exist.

Therefore, the data presented demonstrate that 2 μM CB does not completely inhibit polymerization from the barbed end of an actin filament. Subunit association and dissociation continues to occur, though at a significantly reduced rate. In addition, CB does not behave as a high affinity nucleating factor, nor does it rapidly sever filaments in the absence of shear. A cautionary note is added in that these observations were made under one set of conditions. Given the variation in actin's assembly kinetics and steady state, using different experimental conditions, CB may exhibit different effects if the conditions are varied (2, 4, 18, 29, 39).

Mechanisms of CB-Actin Interaction

Clearly, not enough experimentation has been done to determine quantitatively how the CB-actin interaction leads to a slowing of filament elongation. From the results presented there are at least two possible mechanisms that could explain the slowing of barbed end assembly by CB: subunits can still be added and lost from the barbed end even when CB is bound; and CB is a "poor" competitive inhibitor and elongation results from the net equilibrium between CB binding the barbed end and actin subunits binding the end. Whichever mechanism is correct, it must still be explained why CB completely caps the barbed ends of S_1-decorated filament fragments and the filaments of microvillar cores (28, 33, 39) but not the actin filaments of the acrosomal bundles. Capping could occur from a synergistic effect of CB and the protein(s) bound to those filaments terminal subunits. In the case of the acrosomal actin bundle the synergistic interaction does not exist, and actin polymerization can occur. Another intriguing possibility exists based on the nucleotide (ADP or ATP) bound to the terminal subunits. Brown and Spudich (8) demonstrated that CB has a greater effect on ADP-actin assembly than on ATP-actin assembly. Also, it has long been known that the monomer conformation changes with the binding of these two nucleotides (35). The S_1-decorated filament fragments probably have terminal ADP-subunits, and 2 μM CB does completely cap those filaments preventing monomer addition (28). Likewise, the actin filaments of microvillar cores probably have ADP-actin termini that are capped by CB. From the experiments presented in this report it is clear that 2 μM CB does not completely cap ATP-ends because the terminal subunits of the elongating filaments are ATP-subunits (37, 40). Therefore, the *Limulus* acrosomal actin bundle may be composed of ATP subunits that cannot be capped by CB.

In addition to slowing barbed end assembly, CB also increased the filaments susceptibility to breakage from gentle shear forces. This phenomenon could be due to increased subunit movement within the filament during handling, leading to the exposure of the CB binding site and subsequent breakage. It is now evident that the subunits have a high degree of freedom to rotate, tilt, and bend within the confines of the filament (15, 16, 32, 34, 45). Even the most gentle pipetting could amplify these motions and expose the CB binding site on internal subunits (ADP-subunits?), resulting in filament destabilization and breakage. This hypothesis may provide insight into why not all capping proteins can sever filaments or why they have different rates of severing (2, 3, 9, 20). In conclusion, the internal dynamics of the actin filament, be it the nucleotide bound to the subunit or the movements of the subunits, may prove to be important in regulating the way in which actin binding proteins or cytochalasins interact with actin.

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