Phalloidin Enhances Actin Assembly by Preventing Monomer Dissociation

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ABSTRACT Incubation of the isolated acrosomal bundles of Limulus sperm with skeletal muscle actin results in assembly of actin onto both ends of the bundles. These cross-linked bundles of actin filaments taper, thus allowing one to distinguish directly the preferred end for actin assembly from the nonpreferred end; the preferred end is thinner. Incubation with actin in the presence of equimolar phalloidin in 100 mM KCl, 1 mM MgCl₂, and 0.5 mM ATP at pH 7.5 resulted in a slightly smaller association rate constant at the preferred end than in the absence of the drug (3.36 ± 0.14 x 10⁶ M⁻¹ s⁻¹ vs. 2.63 ± 0.22 x 10⁶ M⁻¹ s⁻¹, control vs. experimental). In the presence of phalloidin, the dissociation rate constant at the preferred end was reduced from 0.317 ± 0.097 s⁻¹ to essentially zero. Consequently, the critical concentration at the preferred end dropped from 0.10 gM to zero in the presence of the drug. There was no detectable change in the rate constant of association at the nonpreferred end in the presence of phalloidin (0.256 ± 0.015 x 10⁶ M⁻¹ s⁻¹ vs. 0.256 ± 0.043 x 10⁶ M⁻¹ s⁻¹, control vs. experimental); however, the dissociation rate constant was reduced from 0.269 ± 0.043 s⁻¹ to essentially zero. Thus, the critical concentration at the nonpreferred end changed from 1.02 μM to zero in the presence of phalloidin. Dilution-induced depolymerization at both the preferred and nonpreferred ends was prevented in the presence of phalloidin. Thus, phalloidin enhances actin assembly by lowering the critical concentration at both ends of actin filaments, a consequence of reducing the dissociation rate constants at each end.

The addition of salt and ATP induces the polymerization of actin in a reaction that involves nucleation and elongation stages (10). Although elongation occurs at both ends of a filament, growth is biased because monomers add to one end at a faster rate than to the other (2, 11). The two ends have been identified by decoration with subfragment 1 of myosin; the “barbed” end is the preferred end for assembly and the “pointed” end is the nonpreferred end for monomer addition (11, 21).

Phalloidin, a highly toxic peptide isolated from the mushroom Amanita phalloides, has been shown to have a marked effect on actin assembly. When phalloidin is added to G-actin before polymerization, it binds to the F-actin that is formed in an ~1:1 molar ratio with actin monomers (20). It has been reported to stabilize actin filaments in liver parenchymal cells (6), increase the extent of actin polymerization (4), lower the critical concentration of actin (i.e., the actin concentration below which no actin assembles) (4), and stabilize F-actin against depolymerization (4, 7, 12). To explain these results, Estes and colleagues (4) have proposed that phalloidin most probably acts by decreasing the dissociation rate constant (k₋₁) rather than by affecting the association rate constant (k₊). It is still not known, however, whether phalloidin affects polymerization at one end (and, if so, at which end) or whether phalloidin affects polymerization at both ends of the actin filaments.

Biochemical techniques used to examine microfilament or microtubule polymerization cannot directly differentiate the reactions at the two ends of the polymer; however, Bergen and Borisy (1) have developed a quantitative morphological method based on the nucleating capacity of flagellar axonemes. Since then, similar assays for microfilament assembly have been developed: one using microvillar cores (11) and another using isolated bundles from the acrosomal processes of Limulus sperm (2, 3, 16). In each system, the ends of the polar seeds can be distinguished morphologically, and nucleated tubules or filaments are distinguishable from the seeds, allowing one to quantitate independently the amount of new growth nucleated at each end. It is possible, by analyzing the rate of filament growth on these seeds as a function of initial actin subunit concentration, to measure both the reaction rate constants as well as the critical concentrations at the two ends of the polymer.

We have used the Limulus bundle assay to determine how
phallolidin affects actin assembly. We have found that within the sensitivity of our assay, the rate constant for association at the preferred end of the filament is slightly lowered in the presence of phallolidin. Moreover, the rate constant of association at the nonpreferred end does not differ in the presence of the drug. However, the critical concentration at each end is drastically reduced and the rate of disassembly at each end as determined by dilution-induced depolymerization is essentially stopped when phallolidin is present. In the presence of phallolidin, therefore, actin assembly is enhanced, not by adding subunits at a faster rate to either end of the filament, but by inhibiting their removal from both ends.

**MATERIALS AND METHODS**

**Terminology:** It should be noted that in keeping with the terminology of Bergen and Borisy (1), we have used the words assembly, elongation, and growth interchangeably to signify the separate molecular events at the end of a filament.

**Organisms:** *Limulus polyphemus* were collected by the Marine Resources Department at the Marine Biological Laboratory in Woods Hole, MA and kept in Instant Ocean Aquaria (Instant Ocean Aquarium Systems, Eastlake, OH) at the University of Pennsylvania.

**Isolation of the Acrosomal Processes of Limulus Sperm:** *Limulus* sperm were collected and the acrosomal bundles isolated according to Tilney (14).

**Preparation of Actin:** Rabbit skeletal muscle actin was prepared in the laboratory of Dr. Annemarie Weber (University of Pennsylvania) according to Spudich and Watt (13) and further purified by the procedures of MacLean-Fletcher and Pollard (8). Actin concentration was determined photometrically at 290 nm using an extinction coefficient of 24,900 M⁻¹ cm⁻¹ (17).

**Actin Assembly Assay:** Gel-filtered actin was diluted to concentrations ranging from 0.02 to 0.14 mg/ml in buffer containing 3 mM Tris, pH 7.5, 0.5 mM ATP, 0.5 mM diethiothreitol, 0.2 mM CaCl₂, and 1.0 mM NaN₃; these samples served as controls. Similar aliquots containing phallloidin (Sigma Chemical Co., St. Louis, MO) in a 1:1 molar ratio with actin served as experimental samples. To each aliquot of 150 μl, 5 μl of a stock solution of *Limulus* bundles suspended in water was added. This stock solution was prepared so that 5 μl *Limulus* stock solution/150 μl assay volume would yield two to three bundles per electron microscope grid space (300 mesh). All solutions had been on ice. At time zero, KCl and MgCl₂ were added to a final concentration of 100 mM and 1 mM, respectively. After mixing, polymerization of actin was allowed to proceed at room temperature. At 2-min intervals, over the next 10 min, taking great care not to shear the bundles with their newly assembled actin filaments, samples were gently pipetted onto electron microscope grids and then stained with 1% aqueous uranyl acetate for 30 s.

In a second experiment to test whether phallloidin affected the rate of depolymerization at the nonpreferred end, actin was again assembled onto *Limulus* bundles in the presence of 100 mM KCl and 1 mM MgCl₂. The concentrations of actin used were selected to assure sufficient growth at the nonpreferred end in the presence or absence of phallolidin. Actin at 0.15 mg/ml was assembled for 15 min to serve as the control. This same amount of actin in the presence of phallolidin resulted in growth so extensive that the filaments splayed out from the ends of the bundles, thus making them difficult to measure accurately. Therefore, actin at 0.03 mg/ml with 1:1 phallolidin served as the experimental sample. Each sample was then diluted with the appropriate buffer (i.e., either with or without phallolidin and containing no salt) in such a way that the resulting actin concentration would be below the critical concentration for the nonpreferred end under each condition (i.e., below 0.04 mM/ml for the control and to negligible amounts for the phallolidin treated sample). This required a fivefold dilution for the control; an 11-fold dilution of the experimental sample was used. After dilution, grids were made every 3 min over a 21-min period, and the amount of disassembly from the nonpreferred end was monitored as a function of time by electron microscopy.

To test whether phallolidin affected the critical concentration and hence, the rate of depolymerization at the preferred end, we added actin in the presence and absence of phallolidin at a concentration determined so that in the control within 15 min, growth at the preferred end of the *Limulus* bundles was extensive but absent from the nonpreferred ends; a different batch of actin was used. After 15 min, the samples were diluted with the appropriate buffer (i.e., either with or without phallolidin) to a concentration which had previously been determined to support no growth on either end of the filaments in the absence of phallolidin. Grids were made every 3 min over a 45-min period and the rate of disassembly was monitored by electron microscopy.

**Electron Microscopy:** Samples were negatively stained with 1% uranyl acetate onto collodion-coated copper grids stabilized by a thin film of carbon and rendered hydrophilic by glow discharge immediately before use. Grids were then examined with a Philips 200 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) at an accelerating voltage of 60 kV. Calibration of the microscope was achieved by using calibration grids from Ernst F. Fullam, Inc. (Schenectady, NY).

**Data Analysis:** Grids at each time point were systematically scanned and only well-stained bundles whose entire lengths were visible were evaluated. The lengths of newly nucleated actin filaments at both the preferred and nonpreferred ends of the bundles were photographed. The negatives were enlarged threefold for a final magnification of x 96 000. Each bundle, depending upon its diameter, can nucleate anywhere from 13 to 100 filaments. Most of the filaments nucleated at each end by a particular bundle are the same length. The average length was used as our estimate of the growth at that end. Therefore, although the number of bundles considered at each time point was normally 12, these values actually represent the average length of a much larger population of filaments.

Elongation (change in filament length / per unit time / t) can be described by two equations (1, 11):

\[ \frac{dl}{dt} = k_2(c) - k_1^* \]

and

\[ \frac{dl}{dt} = k_1^*(c) - k_2^* \]

where \( p \) refers to the preferred end for assembly of an actin filament and \( n \) refers to the nonpreferred end. These equations have the form \( y = mx+b \) (1, 11), so that plotting \( dl/dt \) vs. initial actin concentration \( c_0 \) results in a line whose slope indicates the rate of elongation for that particular end. This was done for a number of protein concentrations. Then, to determine how the rate of assembly varies with protein concentration, each slope and its standard error from the previous set of graphs was plotted vs. initial actin subunit concentration. The slope and standard error of this new line was determined by weighted least squares linear regression. Weighted least squares linear regression gives more emphasis to observations with smaller standard errors and less emphasis to observations with larger standard errors (9). The obtained slope is a measure of the association rate constant, the \( x \)-intercept of each line now corresponds to the critical concentration for that end of the bundle whereas the \( y \)-intercept is a measure of the dissociation rate constant.

A second measure of the rate of disassembly from both the preferred and nonpreferred ends in the presence and absence of phallolidin was obtained by assembling actin onto the bundles (at both ends in one case and at the preferred end only in the second), diluting the sample to below the critical concentration to cause disassembly and monitoring over 3-min intervals by electron microscopy for the time point at which no actin was found at either the preferred or nonpreferred ends (depending on the experiment) of the majority of the bundles.

**RESULTS**

Within the *Limulus* sperm resides a bundle of highly cross-linked and unidirectionally polarized actin filaments (14). This bundle, 50 μm in length, contains 85–100 filaments at one end and gradually tapers to 13 filaments at the other end (15). These bundles can be easily isolated from the sperm and sheared into small segments ranging from 5 to 20 μm in length; they are completely stable in solution. Incubation of these small segments with G-actin under appropriate salt conditions results in actin assembly at both ends of the bundles. In short, the bundles act as stable nucleating centers. As previously observed (2, 3), the newly assembled filaments grow more rapidly at one end than at the other. The amount of new growth is readily recognizable because the newly assembled actin filaments, lacking the bundling proteins present in the *Limulus* sperm, splay out from the ends of the segments (Fig. 1). The assay is very sensitive; growth of as little as 10 monomers in length can be detected. The polarity
FIGURE 1 When *Limulus* bundles are added to a solution of G-actin and the salt concentration is increased to a final concentration of 100 mM KCl and 1 mM MgCl₂, at pH 7.5, actin rapidly assembles onto both ends of the bundles (arrows). Extent of growth is greater at the preferred (+) end of the bundle than at the nonpreferred (−) end. × 31,500.

of new growth is easily learned by measuring at high magnification the diameter of the bundle at each end, because the bundles exhibit taper; the thinner end corresponds to the "barbed" or preferred end for assembly (16). We shall use the notation "+" to represent the barbed end and "−" to represent the pointed or nonpreferred end.

The net rate of filament elongation in the two directions was measured by sampling the reaction of actin monomer with the bundles at two min intervals over a 10-min period. This was done in both the presence and absence of phalloidin. Seven different concentrations of actin ranging from 0.02 to 0.14 mg/ml were examined. At each concentration, a number of bundles were analyzed for growth at each end. Mean lengths ± standard errors were determined, converted to micrometers and plotted vs. time for each concentration of actin (Fig. 2). As can be seen, the filaments grew at constant rates from both ends. The best straight lines were determined by least squares linear regression. Although the plots generally intercepted the x-axis near to zero, short lag periods were sometimes observed. It is not known whether the lag times (average x-intercept was 0.15 min ± 0.16) are significant. Reliable data were difficult to obtain for the preferred end at the higher actin concentrations because growth was extensive and the filaments splayed apart making an accurate measurement of their lengths impossible. On the other hand, at the lower concentrations used, growth at the nonpreferred end was often too little to detect or, of course, nonexistent.

The slopes and standard errors of the time-course plots were then plotted against initial subunit concentrations. These cumulative plots of filament growth rates against monomer concentration for each end could be fitted well with straight lines as determined by weighted least squares linear regression (Figs. 3 and 4). From these same lines we can calculate the association and dissociation rate constants for addition and loss of subunits at the two ends of a filament. As shown by Johnson and Borisy (5) and later applied to analyze each end separately by Bergen and Borisy (1), the slope of this kind of plot provides the association rate constant whereas the y-intercept provides the dissociation rate constant. Each plot intersects the x-axis (where the growth rate is zero) at the critical concentration for that end, i.e., the concentration of actin below which no actin assembles.

As is shown in Fig. 3, the slope of the plot of filament growth-rates as a function of protein concentration at the preferred end was slightly, but statistically, lower in the presence of phalloidin. The slopes, a measure of the association rate constants, were 13.09 ± 0.54 μm/min(mg/ml)⁻¹ vs. 10.13 ± 0.86 μm/min(mg/ml), control vs. experimental. The x-intercepts, a measure of the critical concentration, were 0.004 mg/ml for the control and interpreted as zero for the phalloidin-treated sample (−0.002 mg/ml). The dissociation rate constant given by the y-intercept was −0.052 ± 0.16 μm/min for the control and approached zero for the phalloidin-treated sample (0.02 ± 0.02 μm/min).

Fig. 4 shows the filament growth-rates as a function of protein concentration at the nonpreferred end in the presence and absence of phalloidin. (Notice the change in scale from Fig. 3.) The association rate constant in the presence of phalloidin was indistinguishable from that found for the control (1.01 ± 0.17 μm/min(mg/ml)⁻¹ vs. 1.01 ± 0.06 μm/min(mg/ml)⁻¹, control vs. experimental); however, the critical concentration as measured by the x-intercept was reduced from 0.043 mg/ml in the absence of the drug to zero (−0.001 mg/ml) in its presence. From Fig. 4, we found that the rate constant of dissociation (k₋; as determined by the y-intercept) was greatly reduced from 0.044 ± 0.007 μm/min in the absence of the drug to not different from zero in its presence (0.001 ± 0.007 μm/min). (Table I lists the polymerization constants found.)

To confirm that the k₋ for the nonpreferred end was changed in the presence of phalloidin, we attempted to mea-
Figure 3 Dependence of the rate of elongation on actin subunit concentration at the preferred end of the bundles in the presence (dashed line) and absence (solid line) of 1:1 phalloidin. Each point represents the slope and standard error of a time course such as those shown in Fig. 2. Weighted least squares linear regression results in lines that with extrapolation intersect the x-axis at the critical concentration (C) for this particular end of the bundle. The slope of each line represents the association rate constant. The y-intercepts represent the dissociation rate constants. The slope for the control curve was 13.09 ± 0.54 μm/min/(mg/ml); the x-intercept, 0.004 mg/ml; the y-intercept, -0.052 ± 0.016 μm/min. The slope of the curve for the phalloidin-treated sample was 10.13 ± 0.85 μm/min/(mg/ml); the x-intercept, -0.002 mg/ml; the y-intercept, 0.02 ± 0.02 μm/min.

Figure 4 Dependence of the rate of elongation on actin concentration at the nonpreferred end in the presence (dashed line) and absence (solid line) of phalloidin. Each point represents the slope and standard error of a time course such as those shown in Fig. 2. Weighted least squares linear regression results in growth curves that with extrapolation intersect the x-axis at the critical concentration for this particular end of the bundle. The slope of each line represents the association rate constant. The y-intercept represents the rate of dissociation. The slope for the control sample was 1.01 ± 0.06 μm/min/(mg/ml); the Cc, 0.043 mg/ml; the y-intercept, -0.044 ± 0.007 μm/min. The slope of the line for the phalloidin-treated sample was 1.01 ± 0.17 μm/min/(mg/ml); the Cc, -0.001 mg/ml; the y-intercept, 0.001 ± 0.007 μm/min.

sure directly the rate of depolymerization at the nonpreferred end. Actin at 0.15 mg/ml was assembled for 15 min. This resulted in the mean amount of growth at the nonpreferred end to be 0.76 ± 0.06 μm. The sample was then diluted fivefold, which resulted in the actin monomer concentration becoming below the critical concentration (i.e., <0.043 mg/ml) at the nonpreferred end. Disassembly with time from the nonpreferred end was monitored over 3-min intervals with electron microscopy. In the absence of phalloidin, to quantify the depolymerization rate of the newly assembled actin proved difficult. After 6 min, for example, some of the bundles had completely lost their newly assembled filaments from their nonpreferred end while it appeared that others had lost some but not all of them. Consequently, it was difficult to determine exactly how fast the rate of disassembly was; however, we know that after 15 min, no growth was present at the nonpreferred end of the majority of the bundles observed (Fig. 5). This gave an estimated rate of disassembly of 0.05 μm/min, which is not statistically different from that predicted from Fig. 4. This sample served as the control. In direct contrast, when actin at 0.03 mg/ml was assembled in the presence of phalloidin in a 1:1 molar ratio for 15 min, then with buffer containing phalloidin diluted 11-fold so that the actin concentration would be negligible, even after 21 min, no change in the amount of growth at that end was detectable (0.63 ± 0.01 μm before dilution vs. 0.65 ± 0.02 μm 21 min after dilution). Moreover, the distribution of bundle lengths was so narrow that quantitation was permitted (Fig. 5). (It should be noted again that because, in the presence of phalloidin, the critical concentration at the nonpreferred end was drastically reduced, it was necessary to begin with actin at a concentration lower than that used in the absence of the drug so that growth would not be so extensive and hence, splayed, as to be impossible to measure. Moreover, to observe complete disassembly requires diluting the actin to below the critical concentration for the nonpreferred end in the presence of phalloidin. In that no critical concentration was observed,

\[ k_1 = 3.36 \pm 0.14 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \]
\[ k_2 = 0.26 \pm 0.01 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \]
\[ k_3 = -0.32 \pm 0.10 \text{ s}^{-1} \]
\[ k_4 = -0.27 \pm 0.04 \text{ s}^{-1} \]
\[ C_c = 0.10 \mu M \]
\[ C_l = 0.10 \mu M \]
\[ C_c = 1.02 \mu M \]
\[ C_l = -0.05 \mu M \]

Table I

| Actin Concentration | Control | Phalloidin-Treated |
|---------------------|---------|-------------------|
| 0.03 mg/ml          | 0.001 ± 0.007 μm/min | 0.001 ± 0.007 μm/min |
| 0.15 mg/ml          | 0.10 ± 0.05 μm/min   | 0.05 ± 0.03 μm/min   |

Abbreviations: k1 refers to the association rate constant for the preferred (+) or nonpreferred (-) end of the filament; k2, refers to the dissociation rate constant at these ends; Cc, refers to the critical concentration of actin at either the preferred or nonpreferred end.

* Positive values must be excluded from the range of possibilities.

Inasmuch as negative values are meaningless, these values must be interpreted as essentially zero.
the actin was diluted to a negligible amount, specifically 0.0027 mg/ml.) This supports our conclusion that phalloidin has affected actin assembly at the nonpreferred end by lowering the critical concentration at that end, a consequence of decreasing the dissociation rate constant, to what we interpret as zero.

From Fig. 2, we detected little change in the association rate constant at the preferred end with or without phalloidin. And, in fact, until we regraphed our data using weighted least squares linear regression, which gives more emphasis to values with small standard errors than to those with large standard errors, we had no reason to believe that the critical concentration at the preferred end was altered in the presence of phalloidin. Our last step was to define whether the critical concentration and, hence, the dissociation rate constant at the preferred end are also affected by phalloidin, as we had found for the nonpreferred end. For this experiment, a different batch of actin was used. At the time of its use, it was found to have a high critical concentration; therefore, appropriate controls were performed. Actin was assembled onto Limulus bundles for 15 min with or without phalloidin. The concentration of actin used was that determined in the absence of the drug to result in measurable growth at the preferred end, but in no growth at the nonpreferred end (0.1 mg/ml). After 15 min, the samples were diluted threefold to a concentration determined to be insufficient to support growth at either end in control samples (0.03 mg/ml). Before dilution, the control samples had a average length of 0.885 ± 0.028 µm on the preferred end of the bundles (Fig. 6a). Within 15–21 min after dilution, the majority of the bundles lacked filaments at either end (Fig. 6b). In contrast, in the presence of phalloidin, after 15 min, actin was found to be assembled onto both ends of the actin filaments. Preferred end growth averaged 0.536 ± 0.022 µm whereas growth at the nonpreferred end was 0.411 ± 0.02 µm. Dilution to below the critical concentration for the preferred end in the control sample, resulted in no noticeable disassembly at either end in the phalloidin-treated sample even after 45 min (Fig. 6d). In fact, both ends continued to grow in length (preferred end = 0.673 ± 0.033 µm; nonpreferred end = 0.609 ± 0.06 µm).

These results clearly demonstrate that the critical concentration at the preferred end is also lowered in the presence of phalloidin. In that the association rate constants for the preferred ends with or without phalloidin are only slightly different, we attribute the large decrease in critical concentration to a greatly decreased dissociation rate constant when phalloidin is present. As seen in Fig. 2, the dissociation rate constant appears to be zero.

DISCUSSION

This study using the Limulus bundle assay has measured directly the rate constants for polymerization and critical concentrations at the two ends of the actin filament in the presence and absence of the microfilament-stabilizing drug phalloidin. The method has been used to examine the action of villin (2) and sperm profilin (16) on actin assembly. It is based on the original assay of Bergen and Borisy (1), who used flagellar axonemes as nuclei to study tubulin assembly.

In addition to being able to assess actin assembly at each end of the growing filament independently, the Limulus bundle has the following advantages: (a) ease with which new actin growth can be distinguished from the nuclei, i.e., the acrosomal bundles; (b) sensitivity of the assay since growth as little as 10 monomers long can be detected; (c) taper of the bundles which readily allows one to discern the preferred (thinner) end for actin growth from the nonpreferred (thicker) end for assembly without the use of heavy meromyosin or myosin subfragment-1 decoration of the filaments; and (d) stability of the bundles which do not fall apart in solution.

Although previous studies have examined the mechanism of action of phalloidin on actin, no study has demonstrated whether both or if only one end of the filament is directly affected by the drug. We have found that the association rate constant at the preferred end is only slightly lowered by the addition of phalloidin before initiation of assembly. A possible explanation for this slight decrease can now be offered: because phalloidin lowers the critical concentration at the nonpreferred end, assembly occurs at the nonpreferred end at very low actin concentrations. As a result, locally less monomeric actin may be available for use by the preferred ends, i.e., the preferred ends "see" less actin. This is compounded by the fact that the Limulus bundles taper, thus providing more nonpreferred ends than preferred ends for actin assembly. Hence, locally, the monomer pool is reduced more quickly than in controls, which exhibit no assembly at their nonpreferred ends until the actin concentration exceeds 0.043 mg/ml. Moreover, we have shown by dilution-induced depolymerization that the critical concentration at the preferred end is significantly lowered in the presence of phalloidin. We attribute this large decrease in critical concentration as a result of a decreased dissociation rate constant at this end. It is likely that the off rate is actually zero at the preferred end in the presence of phalloidin.

It is clear from the graphs that the association rate constant at the nonpreferred end does not change in the presence of phalloidin; however, the critical concentration is sharply reduced in the presence of the drug. Our data show this to be a consequence of a decrease in the dissociation rate constant at the nonpreferred end. (A clear possibility is that the off rate is actually zero at this end also.) We have shown that this is the correct interpretation by a second method, namely, by measuring the rate of dilution-induced depolymerization at the nonpreferred end. As expected, the dilution-induced rate of disassembly is markedly reduced in the presence of phalloidin. Actin assembly, therefore, in the presence of phalloidin, occurs at very low actin concentrations. Moreover, once the monomers add, they are unlikely to become removed from either end, thereby stabilizing the actin filaments.

We must point out that the use of weighed least squares linear regression is important when analyzing data using this type of assay, because, in determining regression lines, it minimizes any means with large standard errors. In particular, plots of rate vs. concentration often show points with smaller standard errors near the origin and larger standard errors with increasing concentration. One reason for this may be due to breakage of filaments during specimen preparation when growth is extensive. The problem of determining where the line intercepts the axes is minimized with weighted least squares linear regression. For example, in our first look at our data, we were given no reason to believe that the curves for the preferred end were not identical ± phalloidin. Only after the use of weighed least squares linear regression did we get an indication that the critical concentration at the preferred end was lower and, in fact, likely zero in the presence of phalloidin. This was confirmed in a dilution-induced depolymerization experiment which has proved to be a more
Actin was added to *Limulus* bundles at a concentration that would give growth only on the preferred end of the bundles (a). After 15 min, the samples were diluted threefold which resulted in complete disassembly from the preferred end within 15–21 min (b). However, when the same amount of actin was incubated also for 15 min with bundles, this time, in the presence of phalloidin, actin assembly occurred at both ends of the bundles (c). After dilution of the phalloidin sample as in b, both ends continued to grow. d is at 45 min after dilution. × 99,000.
valuable means for estimating critical concentrations and the rate of disassembly.

Now that we have a clearer view of how phallloidin specifically affects actin assembly, we can confirm and further explain the observations previously made by others. For example, Estes and co-workers (4) showed that phallloidin in equimolar ratio to actin greatly increases the extent of polymerization and significantly reduces the critical concentration. Our results support these claims since the critical concentration is drastically reduced. Moreover, Estes et al. (4) predicted from dilution-induced depolymerization studies that phallloidin acts to block the reverse steps in the polymerization reaction, i.e., decrease the $k_{-1}$. Again, our work supports this conclusion and extends their observation by showing that the critical concentration and depolymerization rate at both ends of actin filaments are greatly reduced in the presence of phallloidin.

In a different study, it was found that, in the presence of phallloidin, actin filaments in liver preparations can be made to assemble (6). Normally, microfilaments are rarely found in liver parenchymal cells, and it is believed that most of the actin in these cells probably exists in the depolymerized state (6). This could be a result of the actin being in too low a concentration to assemble or due to the presence of other proteins which when complexed to actin inhibit its assembly, e.g., profilin. As pointed out by a reviewer, it is possible that phallloidin, by lowering the critical concentration, could shift the equilibrium between actin and profilin-actin to overcome the inhibition and allow assembly.

Phallloidin has been demonstrated to interfere with both cell locomotion and growth (18). Tissue culture cells injected with phallloidin and examined by indirect immunofluorescence microscopy using antibody against actin show dramatic changes in the amount of assembled actin. Moreover, these phallloidin-treated cells show a decrease in their ability to locomote (18). Additionally, cytoplasmic streaming is inhibited in amoebae injected with phallloidin (19). This too, is accompanied by the appearance of extensive arrays of microfilaments. Inasmuch as a precisely regulated arrangement of microfilaments exists in most cells, formation of aberrant microfilaments in inappropriate places, a result of phallloidin addition, will upset the balance. Moreover, the stability of formed microfilaments (a result of their decreased dissociation rate constant) will prevent the filaments from assembling, then disassembling, a mechanism thought to be responsible for cell locomotion.

Now that the mechanism of action of phallloidin has been more clearly defined, it should prove to be a more powerful drug with which to study the role of actin in many biological processes.

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