Mycalolide B, a Novel Actin Depolymerizing Agent*

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We investigated the effects of a novel marine toxin, mycalolide B, on actin polymerization and actin-activated myosin Mg\textsuperscript{2+}-ATPase activity using purified actin and myosin from rabbit skeletal muscle. The results were compared with cytochalasin D which inhibits actin polymerization by binding to the barbed end of F-actin. By monitoring fluorescent intensity of pyrenyl-actin, mycalolide B did not accelerate actin polymerization but quickly depolymerized F-actin, whereas cytochalasin D accelerated actin nucleation and depolymerized F-actin at slower rate. The kinetics of depolymerization suggest that mycalolide B severs F-actin. The relationship between the concentration of total actin and F-actin at different concentrations of mycalolide B suggests that mycalolide B forms 1:1 complex with G-actin. We concluded that mycalolide B severs F-actin and sequesters G-actin and may serve as a novel pharmacological tool for analyzing actin-mediated cell functions.

Mycalolide B was isolated from the marine sponge Mycale sp. by the method described previously (Fusetani et al., 1989). Cytochalasin D and N-(1-pyrenyl)iodoacetamide were obtained from Sigma and Molecular Probes (Eugene, OR), respectively. [γ-\textsuperscript{32}P]ATP was purchased from Amersham (Buckinghamshire, UK).

Preparation of Proteins—Actin was purified from rabbit skeletal muscle (Spudich and Watt, 1971) using buffer G containing 0.2 mM CaCl\textsubscript{2}, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, and 2 mM Tris (pH 8.0 with HCl). Myosin was purified from rabbit skeletal muscle according to the method of Perry (1955). Myosin subfragment-1 (S1) was prepared from purified myosin by the method of Weeds and Taylor (1975).

Actin Labeling—Pyrene labeling was performed by the method described by Wende and Dancker (1986). Briefly, the extracted actin was polymerized with 50 mM KCl for 1 h at 25 °C and diluted to 48 μM. N-(1-pyrenyl)iodoacetamide was then added at final concentration of 48 μM. After pyrene labeling for 30 min at 25 °C, the sample was stored at 0 °C for overnight. The labeled actin was then centrifuged (Hitachi rotor 100AT4) at 48,000 rpm for 3 h, and the pellet was dialyzed for 3 days. Before using the pyrene-labeled actin, the sample was centrifuged again to remove degraded actin.

Viscosity—Viscosity of actin solutions was determined with Ostwald-type viscometers with flow time for water of about 30 s. Specific viscosity (\(\eta_s\)) is defined as \(\eta_s = \eta_{rel} - 1\), where \(\eta_{rel}\) is the flow time for the actin solution divided by the flow time for the corresponding buffer. Measurements of Mg\textsuperscript{2+}-ATPase Activity—The reaction buffer contained 1 μM myosin, 2 mM MgCl\textsubscript{2}, 0.1 mM CaCl\textsubscript{2}, 50 mM KCl, 20 mM Tris (pH 8.8 with HCl), and [γ-\textsuperscript{32}P]ATP (0.3 μ Ci/μl). Mg\textsuperscript{2+}-ATPase activity was monitored by measuring 32P liberated from [γ-\textsuperscript{32}P]ATP (Ikobe and Hartshorne, 1985). Measurements of acto-S1 ATPase activity were performed by method described by Fiske and Subbarow (1925).

Electron Microscopy—Pyrenyl-F-actin (12 μM) was negatively stained with 4% uranyl acetate and observed using a JEOL JEM-1200EX electron microscope at an accelerating voltage of 60 kV.

RESULTS

The effect of mycalolide B on actin polymerization was monitored by measuring fluorescence intensity of pyrenyl-actin. Actin is polymerized in the presence of Mg\textsuperscript{2+} and K\textsuperscript{+}. As demonstrated in Fig. 1A, addition of Mg\textsuperscript{2+} polymerized G-actin after a lag phase. Others have suggested that the lag phase is due to formation of actin dimers or trimers (nucleation), and this is the rate-limiting step for actin polymerization (Nishida and Sakai, 1983). Cytochalasin D decreased the lag phase (acceleration of nucleation) (Huard and Lin, 1979), and it inhibited the final level of polymerization. In contrast, mycalolide B completely inhibited polymerization without accelerating nucleation (Fig. 1A). Fig. 1B shows the concentration-response relationship for the inhibitory effect of mycalolide B and cytochalasin D on actin polymerization. Mycalolide B completely inhibited polymerization whereas cytochalasin D caused a maximal inhibi-

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The abbreviation used are: S1, myosin subfragment-1; acto-S1 ATPase activity; actin-activated S1 Mg\textsuperscript{2+}-ATPase activity.

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The relationship between the total actin concentration and filamentous actin was examined at different concentrations of mycalolide B (Fig. 4A). The lines shifted in a parallel manner as a function of mycalolide B concentration, suggesting increase of the critical concentration. Similar findings have been described for Acanthamoeba profilin (Lal and Korn, 1985) which sequesters G-actin and inhibits polymerization. Fig. 4A also suggests that mycalolide B binds to G-actin in a 1:1 molar ratio, preventing the incorporation of G-actin into filaments. From these results, K<sub>v</sub> value was calculated, by assuming a 1:1 complex of actin with mycalolide B, to be 13–20 nM (see legend for Fig. 4). Since mycalolide B showed a similar concentration dependence in its ability to inhibit G-actin polymerization and to cause F-actin depolymerization, we plotted the amount of increase of the critical concentration where the reaction reached a steady state level at a given concentration of mycalolide B (Fig. 4B). This figure also shows that mycalolide B binds to G-actin in a 1:1 molar ratio.

To confirm if mycalolide B depolymerizes F-actin, viscosity was measured with an Ostwald-type viscometer. As shown in Fig. 5, mycalolide B depolymerized F-actin in a similar manner as the result obtained with pyrenyl-actin (Fig. 2). In this case, however, the rate of depolymerization caused by cytochalasin D was relatively higher than that shown in Fig. 2. This may be because passage through the capillary of viscometer showed similar effect to pipetting in Fig. 2 and thus increased the initial rate of depolymerization.
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Fig. 4. Effects of mycalolide B on fluorescence intensity at different concentrations of actin (4.4% of the total actin had been labeled with pyrene) (A) and on the increase of the critical concentration of actin (B). In A, the fluorescence intensity of actin incorporated into filament was plotted as a function of the total actin concentration in the presence of 5 and 10 μM mycalolide B. F-actin was depolymerized for 120 min in the presence of 5 μM (△) or 10 μM (○) mycalolide B and 0.5% (○) ethanol (control). The intersection on the base line (dashed line) gives a Kc of 13-20 nM assuming a 1:1 complex of actin with mycalolide B is formed. We calculated the following Kd values: 13 nM for 5 μM mycalolide B and 20 nM for 10 μM mycalolide B.

In B, the increase of the critical concentration of actin was plotted as a function of mycalolide B concentration. Increase of the critical concentration was calculated from decrease of fluorescence intensity, since linear relation between fluorescence intensity and actin concentration was obtained from control experiment. G-actin was preincubated for 10 min with mycalolide B before polymerization by adding 1 mM MgCl₂ (○, 26 μM; △, 6.0 μM actin). Alternatively, mycalolide B was added to F-actin polymerized in the presence of 1 mM MgCl₂ (0, 26 μM; △, 22 μM; 4, 20 μM; 7, 15 μM; 10 μM; 7.4 μM; x, 5.0 μM; +, 2.6 μM).

Fig. 5. Effects of mycalolide B and cytochalasin D on F-actin depolymerization assayed by viscometry. Actin (24 μM) was polymerized by adding 0.5 mM MgCl₂ and then mycalolide B or cytochalasin D was added. The abscissa represents time after addition of mycalolide B (○, 1 μM; ●, 10 μM; △, 30 μM) or cytochalasin D (30 μM; □).

were observed. When F-actin were treated with mycalolide B (3 μM; molar ratio to actin = 0.5), only shortened filaments were observed.

Myosin possesses a low level of Mg²⁺-ATPase activity, and this enzymatic activity is enhanced by adding F-actin (Offer et al., 1972) (Fig. 7A). Mycalolide B (30 μM) had no effect on the basal myosin Mg²⁺-ATPase activity in the absence of F-actin, but it completely inhibited the actin-activated myosin Mg²⁺-ATPase activity. Fig. 7B shows that mycalolide B inhibited acto-S1 ATPase activity in a concentration-dependent manner, and the maximum inhibition was obtained when its molar ratio to actin was higher than 1. As shown in Fig. 7C, mycalolide B (10 μM; molar ratio to actin = 1.4) decreased viscosity of F-actin in the presence of S1. These results suggest that mycalolide B depolymerizes actin in the presence of myosin and this may be the mechanism of inhibition of actin activation of myosin ATPase activity.

Finally, we examined the effect of mycalolide B in the presence of phalloidin, which binds to F-actin in a 1:1 stoichiometry and inhibits dissociation of G-actin from both ends of F-actin (Sampath and Pollard, 1991). Phalloidin (12 μM) almost completely inhibited the depolymerization induced by 10 μM mycalolide B (Fig. 8).
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FIG. 7. Effects of mycalolide B on actomyosin or acto-S1. A, effects of mycalolide B and cytochalasin D on myosin Mg²⁺-ATPase activity either in the absence or presence of actin. The concentration of released inorganic phosphate was measured at 25 °C with (closed symbols) or without (open symbols) 12 μM actin. Mg³⁺-ATPase activity is represented by the slopes of the lines. The reaction mixtures were preincubated with either mycalolide B (30 μM) or cytochalasin D (30 μM) for 10 min before adding 1 mM [γ-32P]ATP. The activities without toxins (♦, □) were the same as those in the reaction mixture containing 0.5% ethanol which was the maximal concentration when the highest quantities of toxins were added. B, effects of different concentration of mycalolide B on acto-S1 ATPase activity. The concentration of released inorganic phosphate was measured at 25 °C in the presence of 2.4 μM actin and 1 μM S1. The reaction mixtures were preincubated with mycalolide B (300 nM to 30 μM) for 10 min before adding 1 mM ATP. Mg²⁺-ATPase activity is plotted as a function of molar ratio of mycalolide B/actin. C, change in viscosity by mycalolide B of F-actin in the presence of actin. Mg²⁺-ATPase activity is plotted as a function of molar ratio (severing activity) as shown in Fig. 4. The present results demonstrated that mycalolide B binds G-actin and actively depolymerizes it, forming the G-actin-mycalolide B complex with a 1:1 molar ratio (severing or nibbling). When mycalolide B binds to G-actin, this complex never polymerizes even in the presence of Mg²⁺ (monomer sequestration).

FIG. 8. Effect of phalloidin on mycalolide B-induced depolymerization. Experimental conditions are the same as those described in Fig. 2. Mycalolide B (10 μM) was applied to F-actin in the presence (△) or absence (△) of 12 μM phalloidin.

FIG. 9. Schematic representation of the mode of action of mycalolide B (●) and cytochalasin D (●) on actin. Each trapezium represents actin molecule. Cytochalasin D caps the barbed end of F-actin and allows its depolymerization from the pointed end (capping). Cytochalasin D binds to G-actin and forms the complex of two G-actins with one molecule in the presence of Mg²⁺ (Goddette and Frieden, 1986) (nucleation ↑). Mycalolide B binds to F-actin and actively depolymerizes it, forming the G-actin-mycalolide B complex with a 1:1 molar ratio (severing or nibbling). When mycalolide B binds to G-actin, this complex never polymerizes even in the presence of Mg²⁺ (monomer sequestration).

DISCUSSION

The present results showed that mycalolide B is the depolymerizing agent, and this mechanism is quite different from that of cytochalasin D. Stossel (1989) classified actin-binding proteins with their mode of action. "Severing" proteins, such as gelsolin or villin, block the barbed ends of actin filaments and promote nucleation. Depactin, actophorin, desmin, and actokinin are categorized as "nibbling" proteins, which sever F-actin and bind to G-actin in stoichiometry with no capping activity nor promotion of nucleation. However, the nibbling proteins accelerate the rate of polymerization since they never F-actin into short filaments and increase the number of nuclei for polymerization (Maciver et al., 1991; Mabuchi, 1983; Nishida et al., 1984). Profilin, which controls monomer polymerizability (Stossel, 1988), binds only to G-actin and decreases polymerization rate by sequestering G-actin (Pollard and Cooper, 1984). Present results demonstrated that mycalolide B binds G-actin with 1:1 (monomer sequestering activity) (Fig. 4) with no promotion of nucleation (Fig. 1). However, its depolymerizing effect cannot be explained by simple monomer sequestering activity as shown in Fig. 3 that mycalolide B increased filament number. Janmey et al. (1985) showed the different time course of actin depolymerization between actin-severing protein (gelsolin) and G-actin-sequestering protein (vitamin D-binding protein) after dilution (Janmey et al., 1985), and we also conclude that mycalolide B possesses severing activity. In this respect, mycalolide B seems to act on actin like nibbling proteins. Unlike the nibbling proteins, however, mycalolide B does not accelerate the rate of polymerization. The differences in the mode of action between mycalolide B and nibbling proteins may be explained by that mycalolide B would be chelated by G-actin because of the strong monomer binding activity. Alternatively, the severing activity of mycalolide B is weaker than nibbling...
proteins. On the other hand, it is known that cytochalasin D caps the barbed end of F-actin (Sampath and Pollard, 1991) and also promotes nucleation (Howard and Lin, 1979). Therefore cytochalasin D acts on actin like capping proteins such as β-actinin.

Mycalolide B inhibited actin-activated myosin Mg²⁺-ATPase activity. In contrast, cytochalasin D, even at 30 μM, did not inhibit actin-activated myosin Mg²⁺-ATPase activity (Fig. 6A). The lack of the inhibitory effect of cytochalasin D on actin-activated myosin-ATPase activity may be attributable to the incomplete depolymerization of F-actin by cytochalasin D (Howard and Lin, 1979) (see Fig. 1B). F-actin, which has been shortened by cytochalasin D, may still activate myosin Mg²⁺-ATPase activity.

The present results further demonstrated that the mycalolide B-induced depolymerization was completely suppressed by phalloidin (Fig. 6). Phalloidin has been suggested to bind F-actin and block the dissociation of actin monomer from both ends of F-actin (Sampath and Pollard, 1991). Stoichiometry of its binding for filaments is one phalloidin to one actin protomer and the binding causes conformational change of F-actin (Sampath and Pollard, 1991). Further investigations are needed to know whether phalloidin inhibits the binding of mycalolide B or blocks the cleavage of actin filament.

Mycalolide B had no effect on the polymerization of tubulin isolated from swine brain (data not shown), the Mg²⁺-ATPase of skeletal muscle myosin alone (Fig. 6A), the phosphorylation of smooth muscle myosin regulatory light chain, or cytosolic Ca²⁺ concentrations in smooth muscles (Hori et al., 1993), suggesting that mycalolide B affects the contractile apparatus in a rather specific manner, i.e. via depolymerization of F-actin.

Recently, two natural products were identified and found to act on actin. Patterson et al. (1993) demonstrated that tolytxin, a macrolide isolated from cyanobacteria with resembled chemical structure with mycalolide B (Ishibashi et al., 1986; Fusetani, 1987), inhibits actin polymerization and disrupts cytoskeletal conformation in L1210 cells. However, the precise mechanism of its action on actin has not yet been revealed. Furukawa et al. (1993) demonstrated that goniodomin A, another macrolide isolated from dinoflagellate, activates (at lower concentration) or inhibits (at higher concentration) actomyosin Mg²⁺-ATPase activity. Higher concentration of goniodomin A reduces fluorescence of pyrene labeled actin. However, the mode of action of goniodomin A seems to be different from mycalolide B, since goniodomin A does not change the length of F-actin.

In conclusion, (i) mycalolide B binds to G-actin with a 1:1 molecular ratio, (ii) depolymerizes actin filaments at rates that exceed the maximal rate of depolymerization achieved by cytochalasin D, and (iii) inhibits polymerization of G-actin. These effects are different from the capping effect of cytochalasin D, which only shifts the polymerization-depolymerization equilibrium to decrease G-actin (see Fig. 9). Mycalolide B is a "depolymerizing" agent, and it is a novel and potentially important tool for studies of actin-mediated cell functions.

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