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Actin-Depolymerizing Effect of Dimeric Macrolides, Bistheonellide A and Swinholide A[1]

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We compared the effects of dimeric marine toxins, bistheonellide A, and swinholide A, on actin polymerization. Bistheonellide A and swinholide A possess two identical side chains with similar structures to those of other marine toxins, mycalolide B, and aplyronine A. By monitoring changes in fluorescent intensity of pyrenyl-actin, bistheonellide A was found to inhibit polymerization of G-actin and to depolymerize F-actin in a concentration-dependent manner. The relationship between the concentration of bistheonellide A and its inhibitory activity on actin polymerization suggested that one molecule of bistheonellide A binds two molecules of G-actin. We demonstrated by SDS-PAGE that the complex of G-actin with bistheonellide A, swinholide A, or mycalolide B could not interact with myosin. No evidence was found that bistheonellide A severs F-actin at the concentrations examined (molar ratio to actin; 0.025-2.5), while swinholide A showed severing activity, although it was weaker than that of mycalolide B. We also demonstrated that the depolymerizing effect of bistheonellide A or mycalolide B is irreversible. Bistheonellide A increased, while swinholide A decreased, the rate of nucleotide exchange in G-actin, suggesting that binding of these toxins induces different conformational changes in the actin molecule. These results suggest that bistheonellide A intervenes between two actin molecules, forms a tertiary complex with each of its side chains bound to G-actin, and inhibits polymerization by sequestering G-actin from incorporation into F-actin. A difference in structure at the end of the side chain between dimeric macrolides and mycalolide B may account for the weak severing activity of the former.

Key words: actin depolymerization, bistheonellide A, dimeric macrolide, mycalolide B, swinholide A.

Bistheonellide A is a novel macrolide isolated from the marine sponge *Theonella* sp., which was found at Hachijo-jima Island, Japan (*1*). Bistheonellide A is a cytotoxic to L1210 cells (*2*) and disrupts stress fibers to elicit morphological changes in the rat fibroblast at submicromolar concentrations (*3*).

Previously, we have reported that the marine toxin mycalolide B, isolated from the marine sponge *Mycale* sp., bound to G-actin in a 1:1 molar ratio and actively, selectively, and completely depolymerized F-actin to G-actin by its severing effect (*4*). The depolymerizing action of mycalolide B is comparable to the action of such endogenous "nibbling proteins" (*5*) as depactin (*6*), destrin (*7*), or actophorin (*8*), but distinct from the capping activity of cytochalasins (*9-11*). Furthermore, cytochalasin D (*4, 9-11*) but not mycalolide B (*4*) promotes actin nucleation. We also found that a novel marine toxin, aplyronine A, isolated from the sea hare *Aplysia kurodai*, is similar in the structure and effect to mycalolide B (*12*).

Bistheonellide A is a unique macrolide having a dimeric structure. Each monomeric unit contains a motif which is common to mycalolide B or aplyronine A (Fig. 1). Recently, Bubb *et al.* (*13*) have shown that a dimeric macrolide, swinholide A, severed F-actin, and bound to G-actin in a 1:2 molar ratio. The structure of swinholide A is similar to that of bistheonellide A (Fig. 1), possessing two identical side chains. Each chain resembles that of mycalolide B and aplyronine A. To determine the structures of macrolide that are responsible for the binding and sequestering of G-actin, and the severing of F-actin, we compared the inhibitory effects on actin of bistheonellide A, swinholide A, and mycalolide B.

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Abbreviations: pyrenyl-actin, pyrene-labeled actin; εATP, 1,N^6-ethenoadenosine 5′ triphosphate; G-ADP-actin, G-actin binding ADP; F-ADP-actin, F-actin polymerized from G-ADP-actin.

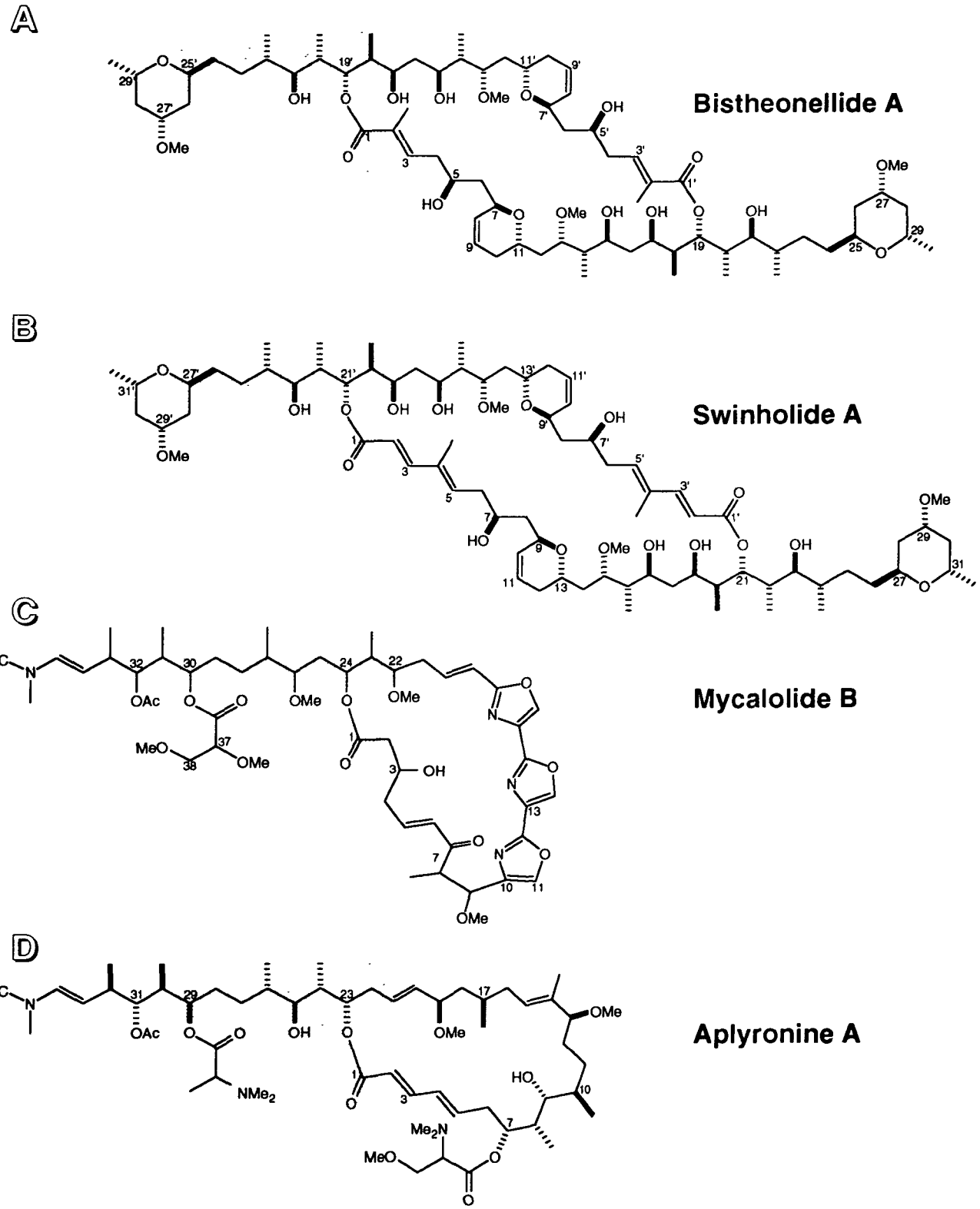

Fig. 1. **Chemical structures of actin depolymerizing marine macrolides.** A, bistheonellide A; B, swinholide A; C, mycalolide B; D, aplyronine A. The common motif in the four macrolides is shaded: C-19 to C-25 of bistheonellide A, C-21 to C-27 of swinholide A, C-24 to C-30 of mycalolide B, C-23 to C-29 of aplyronine A, and the ester structure involving C-1 of the macrolide ring.

MATERIALS AND METHODS

Chemicals—Bistheonellide A (*1*), swinholide A (*14*), and mycalolide B (*4*) were isolated from the marine sponges *Theonella* sp., *Theonella swinhoei*, and *Mycale* sp., respectively, as described previously. Cytochalasin D was obtained from Sigma Chemicals (St. Louis, MO). *N*-(1-Pyrene)iodoacetamide and 1,N^6-ethenoadenosine 5′ triphosphate (ε ATP) were obtained from Molecular Probes (Eugene, OR). All other chemical were of analytical grade.

Preparation of Proteins and Measurement of Superprecipitation—Actin was extracted from acetone powder of rabbit fast skeletal muscle using buffer G containing 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, and 2 mM Tris-HCl (pH 8.0) (*15*). Crude G-actin was further purified by Sephadex G-200 gel filtration. G-Actin which had been dialyzed against buffer G containing 0.2 mM ADP instead of ATP was used as F-ADP-actin. Myosin was purified from fast rabbit skeletal muscle as described by Perry (*16*). G-Actin (1 μM) was polymerized and diluted in a solution containing 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 20 mM Tris-HCl (pH 7.5). F-Actin thus prepared was treated with bistheonellide A (1 μM), swinholide A (1 μM), mycalolide B (1 μM), cytochalasin D (1 μM), or vehicle control (1.3% DMSO) for 1 h at 25°C. Consequently, myosin (1 μM) in an equal volume of the same solution was added to the actin mixture in the presence of 1 mM ATP and kept at 25°C for 30 min. Actomyosin suspension thus prepared was centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant and precipitate were subjected to SDS-PAGE (*17*).

Measurement of Polymerization of Fluorescent Pyrenyl-Actin—Pyrene labeling was performed by the method of Wendel and Dancker (*18*) with slight modification as described previously (*4*). The time course of polymerization initiated by the addition of 50 mM KCl and 2 mM $MgCl_2$ to buffer G was continuously monitored by measuring the fluorescence of pyrenyl-actin (2.5% of total actin) with a fluorometer (FP2060, JASCO, Tokyo; excitation at 365 nm and emission at 407 nm) at 25°C. Depolymerization of F-actin with marine toxins was monitored under similar conditions.

Measurement of Binding of Bistheonellide A to G-Actin—Binding assay was performed as described by Goddette and Frieden (*19*) with slight modifications. Briefly, G-actin in buffer G was incubated in a temperature-controlled water-bath at 25°C for 10 min. Bistheonellide A in various concentrations was added to the G-actin solution, then 500-μl aliquots were immediately taken and loaded onto an Amicon Centrifree micropartition filter. The filters were spun at 1,000 × *g* for 10 min at 25°C, and concentrations of bistheonellide A in 100-μl samples of the ultrafiltrates were determined by HPLC with a Superiorex ODS reverse phase column (Shiseido, Tokyo) with 80% acetonitrile. The eluate was monitored for absorbance at 240 nm, and bistheonellide A concentration was determined by comparing the peak with those of standard solutions.

Nucleotide Exchange Assay—G-actin solutions were diluted 100 times with a buffer containing 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 2 mM Tris-HCl (pH 8.0) 10 min before addition of 50 μM ε ATP. The fluorescence of ε ATP was monitored with a fluorometer (FP2060, excitation at 360 nm and emission at 410 nm) at 25°C (*20*).

RESULTS

Inhibitory Effects of Bistheonellide A on Actin Polymerization—Figure 2A shows the inhibitory effects of bistheonellide A on actin polymerization. Bistheonellide A was added to G-actin (6 μM), and 12 min later polymerization was started by the addition of 50 mM KCl and 2 mM $MgCl_2$. In the absence of bistheonellide A, the actin polymerization process showed a lag phase followed by elongation and steady state phases. Bistheonellide A inhibited the steady level of actin polymerization and the rate of elongation in a concentration-dependent manner. Bistheonellide A did not shorten the lag phase (Fig. 2A). Figure 2B shows the effects of bistheonellide A, swinholide A, mycalolide B,

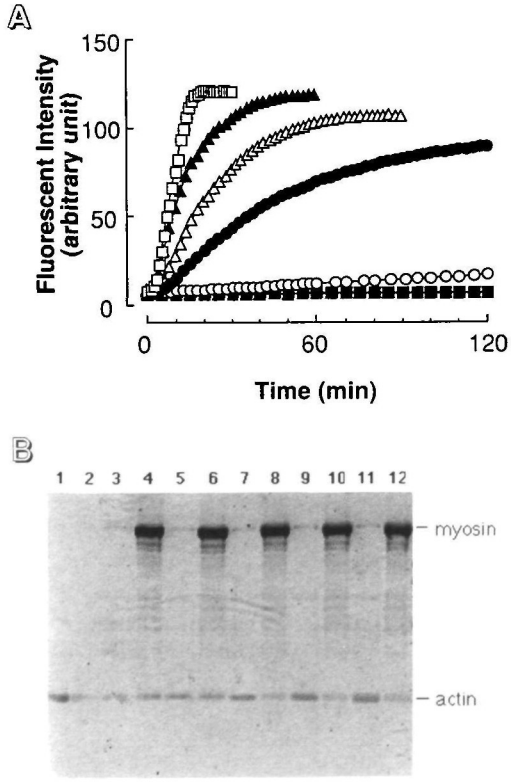

Fig. 2. **The inhibitory effect of bistheonellide A on actin polymerization.** A: 6 μM actin was incubated in buffer G 12 min before polymerization at 25°C in the presence or absence of bistheonellide A. At time 0, 50 mM KCl and 2 mM $MgCl_2$ were added and polymerization was monitored with a fluorometer. Concentrations of bistheonellide A were 0 μM (□), 0.05 μM (▲), 0.2 μM (△), 1 μM (●), 1.5 μM (○), and 3 μM (■). B: The effect of bistheonellide A on cosedimentation of actin with myosin. Actin (1 μM) was treated with 1 μM cytochalasin D (lanes 5 and 6; relative amounts were 0.16 and 0.17, respectively), 1 μM bistheonellide A (lanes 7 and 8; relative amounts were 0.20 and 0.13, respectively), 1 μM swinholide A (lanes 9 and 10; relative amounts were 0.22 and 0.12, respectively), 1 μM mycalolide B (lanes 11 and 12; relative amounts were 0.22 and 0.10, respectively) or without compounds (lanes 3 and 4; relative amounts were 0.08 and 0.13, respectively) for 1 h at 25°C. Subsequently myosin (1 μM) and ATP (1 mM) were added (except lanes 1 and 2; relative amounts were 0.22 and 0.11, respectively), and 30 min later samples were centrifuged at 10,000 × *g* for 10 min. Resulting supernatants (lanes 1, 3, 5, 7, 9, and 11) and precipitates (lanes 2, 4, 6, 8, 10, and 12) were applied to SDS-PAGE.

and cytochalasin D on the binding of actin with myosin in the presence of ATP under superprecipitation conditions. Pretreatment of F-actin with cytochalasin D (lanes 5 and 6) had no effect. On the other hand, bistheonellide A (lanes 7 and 8), swinholide A (lanes 9 and 10), and mycalolide B (lanes 11 and 12) increased the amount of actin unbound to myosin and left in the supernatant (lanes 7, 9, and 11).

Stoichiometry of Bistheonellide A Bound to Actin—As shown in Fig. 3A, the intensity of F-actin fluorescence was proportional to actin concentration. The critical concentration of actin for polymerization was calculated to be 0.98 μM. Bistheonellide A (1 μM) shifted the relationship between the fluorescence intensity and actin concentration to the right in a parallel manner and increased the observed critical concentration from 0.98 to 2.94 μM. The increment of the critical concentration (1.96 μM) was larger than the concentration of bistheonellide A added (1 μM). The K_d value of 0.152 μM was determined by the equation,

$$K_d^2 = [\text{BT-A}] \cdot c^2 / [\text{A}_2\text{B}] \quad (1)$$

assuming highly cooperative binding of two actin molecules (see below) to bistheonellide A ($[\text{A}_2\text{B}] \gg [\text{AB}]$); c for the actin critical concentration, [BT-A] for the concentration of free bistheonellide A (total minus $[\text{A}_2\text{B}]$), and $[\text{A}_2\text{B}]$ and [AB] for the concentrations of bistheonellide A bound to two and to one actin molecule, respectively.

Figure 3B displays the concentration-inhibition relationship of bistheonellide A with actin polymerization. In the first series of experiments, bistheonellide A was added to F-actin and the final level of depolymerization was plotted as a function of the molar ratio of bistheonellide A to F-actin (Fig. 3B, closed symbols). In the next series of experiments, bistheonellide A was added to G-actin and consequently polymerization was started. The final level of polymerization was plotted as a parameter for inhibition by bistheonellide A (Fig. 3B, open symbols). In both series of experiments, bistheonellide A completely inhibited actin polymerization at a concentration of approximately half that of the F-actin fraction. It should be noted that the molar ratio of bistheonellide A to actin was plotted with the concentration of actin in F-form (total minus the critical concentration, 0.98 μM; see Fig. 3A), rather than total actin. We calculated the K_d value to be 0.355 μM from Eq. (1).

We further examined the binding of bistheonellide A to G-actin at a concentration of 3.7 μM. As shown in Fig. 3C, it was saturated at approximately 2 μM. The number of binding sites in bistheonellide A to G-actin, n, was calculated to be 1.7 and the apparent binding constant K_d to be 0.15 μM, assuming the presence of positive cooperativity, as assessed by Hill's equation:

$$\log \frac{B}{B_{max} - B} = \frac{1}{n}(\log F - \log K_d) \quad (2)$$

where B is the concentration of bistheonellide A bound to G-actin ($[\text{A}_2\text{B}]$) and B_{max} is the highest concentration of bound bistheonellide A (*i.e.*, [total actin]/n). F is the concentration of free bistheonellide A ([BT-A]), K_d is the dissociation constant for bistheonellide A to G-actin, and n is the number of binding sites on G-actin to bistheonellide A.

Kinetics of Actin Depolymerization by Bistheonellide A—Figure 4A compares the time-courses of actin depolymerization induced by bistheonellide A, swinholide A, and cytochalasin D. Swinholide A (1 μM) rapidly depolymerized 4 μM F-actin, suggesting the presence of a severing effect (*13*). In contrast, cytochalasin D slowly and monotonically depolymerized F-actin. The time-course of 1 μM bistheonellide A-induced depolymerization was slower than that of swinholide A, but faster than that of cytochalasin D even at excessive concentration (5 μM). We also

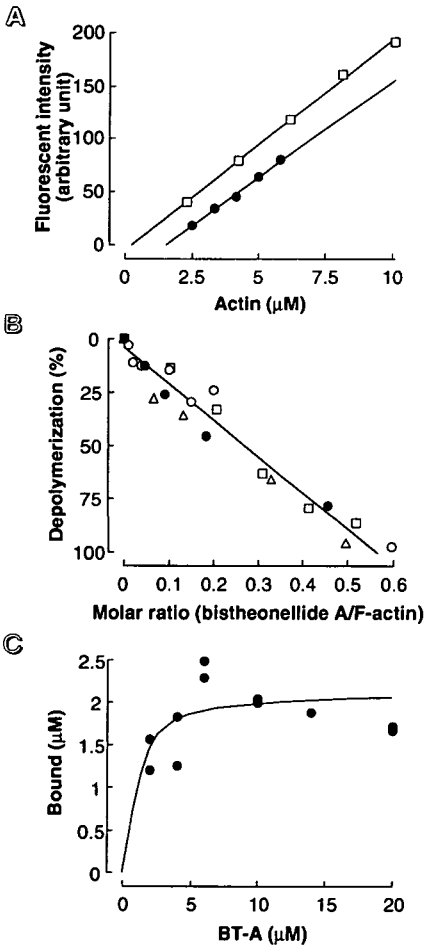

Fig. 3. **A: Effect of bistheonellide A on fluorescent intensity of actin.** The fluorescent intensity of actin incorporated into filaments was plotted as a function of the total actin concentration in the absence (□) or presence (●) of 1 μM bistheonellide A. F-Actin was depolymerized for 4 h in the presence of bistheonellide A. **B: Inhibition of actin polymerization as a function of molar ratio of bistheonellide A to F-actin fraction.** Bistheonellide A was added to F-actin (12 μM, ●) or to G-actin (5 μM, □; or 6 μM, ○) and the degree of depolymerization was calculated from the fluorescence intensity at steady state (120 min). The ordinate depicts percentage depolymerization, where fluorescence intensity of F-actin in the absence of bistheonellide A was taken as 0% and fluorescence intensity of G-actin (before polymerization) was taken as 100%. The abscissa represents the molar ratio of bistheonellide A to F-actin. **C: Binding of bistheonellide A to 3.7 μM actin in buffer G.** After incubation for 10 min, bistheonellide A bound to actin was removed by filtration, and the concentration of free bistheonellide A in the filtrate was measured by HPLC as described in "MATERIALS AND METHODS." The abscissa represents the concentration of applied bistheonellide A, and the ordinate represents the concentration of bistheonellide A trapped by the G-actin fraction. The curve was generated by fitting the data to the Hill's equation.

compared the effect of bistheonellide A with that of swinholide A on actin polymerization (Fig. 4B). Although the final level of polymerization was the same, polymerization of swinholide A-treated actin was faster than that of bistheonellide A-treated actin.

The effects of bistheonellide A and swinholide A on F-actin are shown in Fig. 5A. The effect of mycalolide B is also shown for comparison. F-Actin was diluted with buffer G to a final concentration of 120 nM in the presence and absence of each compound (60 nM bistheonellide A, 60 nM swinholide A, or 120 nM mycalolide B) and spontaneous decrease of the fluorescent intensity was then monitored. Since the velocity of the decay of the fluorescent intensity is proportional to the number concentration of F-actin, shortening of the half-time of the decrease of the fluorescent intensity indicates an increment in the number concentration of F-actin by severing effects (*4*). Figure 5A indicates that bistheonellide A did not increase the number concentration of F-actin at the concentration examined.

In the next experiment, we examined the concentration-response relationship of these compounds in severing F-actin (Fig. 5B). F-Actin was diluted with buffer G to a final concentration of 30 nM in the presence of various concentrations of macrolides. Mycalolide B most rapidly shortened the half-time of decay of the fluorescent intensity by dilution, even in a molar ratio to actin of less than 0.5. Swinholide A also decreased the half-time. However, bistheonellide A was almost ineffective (Fig. 5B). Furthermore, we compared the severing effect of mycalolide B with those of bistheonellide A and swinholide A (Fig. 5C). F-Actin was diluted with buffer G to a final concentration of 20

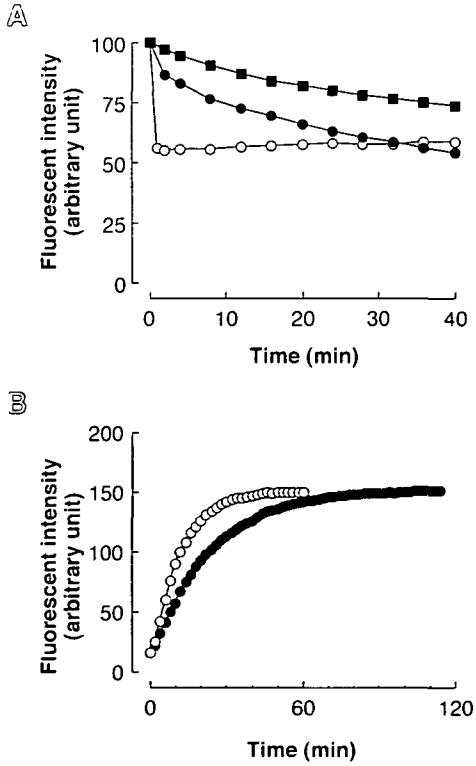

Fig. 4. **Inhibitory effects of bistheonellide A, swinholide A, and cytochalasin D on F-actin.** A: Bistheonellide A (●, 1 μM), swinholide A (○, 1 μM), or cytochalasin D (■, 5 μM) was applied to F-actin (4 μM) at time 0 and the decrease of fluorescence intensity was monitored. B: Comparison of inhibitory effects between bistheonellide A and swinholide A on actin polymerization. Actin (12 μM) was incubated with 3 μM bistheonellide A (●) or swinholide A (○) for 15 min followed by polymerization.

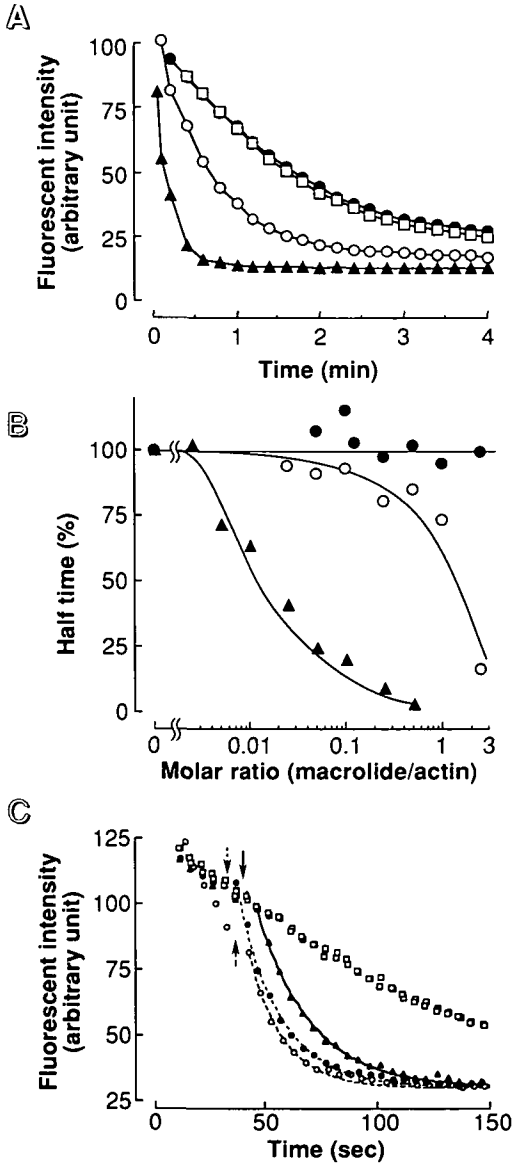

Fig. 5. **Comparison of the severing activities of bistheonellide A and other macrolides.** A: Spontaneous depolymerization in unpolymerizable state. F-Actin (12 μM) was diluted 100 times with buffer G in the absence (□) or presence of 60 nM bistheonellide A (●), 60 nM swinholide A (○), or 120 nM mycalolide B (▲). B: Macrolide concentration-dependency of severing activity in terms of half-time of depolymerization. F-Actin (5 μM) was diluted 250 times with buffer G in the presence of each concentration of bistheonellide A (●), swinholide A (○), or mycalolide B (▲). Each depolymerization curve was fitted to a single exponential. The half-time in the presence of a macrolide relative to that in its absence is plotted against the concentration of macrolide. C: F-Actin (5 μM) was diluted 250 times with buffer G in the absence (▲) or presence of 20 nM bistheonellide A (●) or swinholide A (○), then 5 nM mycalolide B was added (arrows for each sample). The decay of fluorescent intensity with time is plotted for control (solid line), bistheonellide A (dotted line), and swinholide A (broken line) after addition of mycalolide B and in the absence of macrolide (□, 2 samples).

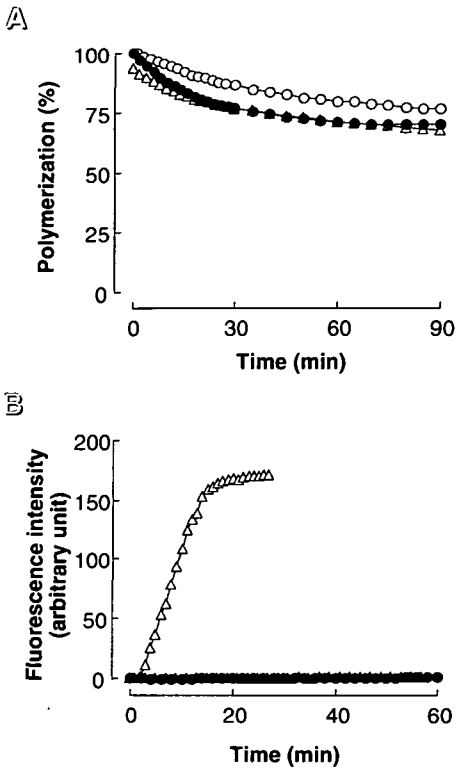

Fig. 6. **A: Effect of bistheonellide A and cytochalasin D on F-ADP-actin.** Polymerization of 9.45 μM G-ADP-actin was induced by adding 50 mM KCl and 2 mM $MgCl_2$. When polymerization reached a steady state (after 80 min), 0 μM (○), 50 nM (●), or 1 μM (△) cytochalasin D was added; then 60 min later, 1 μM bistheonellide A was added. The ordinate depicts percentage polymerization, where the fluorescent intensity of G-actin (before polymerization) was taken as 0% and that at the time of addition of bistheonellide A (time=0) was taken as 100%. **B: Effect of dialysis on actin-macrolide mixture.** A 5.2 μM solution of F-actin (1 ml) was depolymerized with 2.5 μM bistheonellide A, 5 μM mycalolide B, or by sonication. After fluorescent intensity had decreased to a level similar to that of G-actin, each sample was dialyzed against 100 ml of buffer G overnight at 4°C, then 50 mM KCl and 2 mM $MgCl_2$ were added (time=0) and fluorescent intenstiy was monitered.

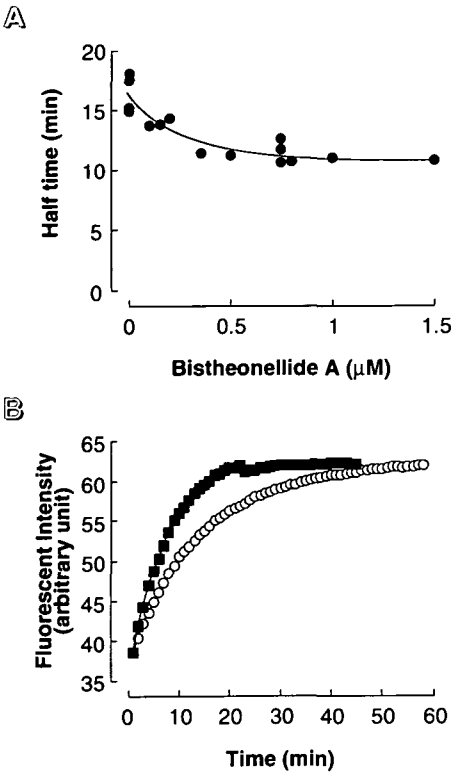

Fig. 7. **Effects of bistheonellide A and swinholide A on the exchange of εATP with ATP bound to G-actin.** A: Dependence of the half-time of the exchange reaction on bistheonellide A concentration. εATP (50 μM) was added to 1.4 μM ATP-actin in buffer G without ATP at various concentrations of bistheonellide A. B: Inhibitory effects of swinholide A on εATP exchange rate. Actin (0.6 μM) was incubated for 10 min in the absence (■) or presence (○) of 0.3 μM swinholide A. Then 50 μM εATP was added (time=0) and fluorescent intensity was monitored.

nM in the absence or presence of 20 nM bistheonellide A or swinholide A. Then mycalolide B (final concentration of 5 nM) was added and the increment in the rate of depolymerization was observed. The mycalolide B-induced depolymerizing rate was unchanged by the presence of bistheonellide A or swinholide A.

Carlier *et al.* (*21*) reported that when actin polymerized in the presence of ADP instead of ATP, the critical concentrations of barbed end and pointed end become similar and cytochalasin looses its inhibitory effect on actin polymerization. As shown in Fig. 6A, bistheonellide A depolymerized F-ADP-actin. At 50 nM, cytochalasin D had no effect on actin polymerization but inhibited the rate of bistheonellide A-induced depolymerization. At 1 μM, cytochalasin D depolymerized actin slightly (6.3%).

Next, 5.2 μM F-actin was depolymerized with either 2.5 μM bistheonellide A, 5 μM mycalolide B, or by sonication, then each sample was dialyzed against 100 volumes of buffer G overnight at 4°C. These samples were again treated with 50 mM KCl and 2 mM $MgCl_2$. As shown in Fig. 6B, only the sonicated sample retained the capacity to polymerize.

Effect of Bistheonellide A on the Nucleotide Exchange Rate in G-Actin—Nishida (*22*) demonstrated that the G-actin-sequestering proteins change the rate of nucleotide exchange in G-actin. We investigated such effect of bistheonellide A by probing with a fluorescent analog of ATP, εATP. Figure 7A shows that the binding of bistheonellide A increased the nucleotide exchange rate. On the other hand, swinholide A suppressed the rate (Fig. 7B), as reported for mycalolide B and aplyronine A (*12*).

DISCUSSION

In this study, we found that bistheonellide A inhibited actin polymerization in a concentration-dependent manner. Bistheonellide A also increased the critical concentration of actin for polymerization to a level higher than the concentration of added bistheonellide A, but lower than the twice the added bistheonellide A concentration (Fig. 3A). Figure 3B shows that bistheonellide A inhibits actin polymerization, at a concentration of half that of the F-actin fraction. We also performed binding assay using ultrafiltration and HPLC as described by Goddette and Frieden (*19*). The binding curve of bistheonellide A with G-actin indicated

saturation at a concentration of approximately half that of the G-actin concentration (Fig. 3C). Therefore, it may be concluded that one molecule of bistheonellide A binds two G-actin molecules to form a tertiary complex. The variation in K_d values for the binding of bistheonellide A to G-actin (0.152 μM from Fig. 3A, 0.355 μM from Fig. 3B, and 0.15 μM from Fig. 3C) may be due to differences in ionic conditions and/or the conformation of the actin molecule under various assay conditions, and experimental error. For comparison, from the results of analytical ultracentrifugation, Terry *et al.* reported a K_d of 0.050 μM for misakinolide A (*23*) and Bubb *et al.* suggested 0.10 μM for swinholide A (*13*).

As shown in Fig. 2A, bistheonellide A did not shorten the lag phase of polymerization. Aplyronine A (*12*) and mycalolide B (*4*) also did not shorten the lag phase, suggesting that these compounds do not promote actin nucleation. The actin-bistheonellide A complex is thus thought to differ in conformation from the actin dimer binding with cytochalasin D (*19, 24*) or the native actin dimer, because only the latter two forms serve as nuclei for actin polymerization and shorten the lag phase (*24, 25*). In the presence of ATP, F-actin, but not G-actin (*26*), interacts with myosin, and the two cosediment on centrifugation at a low gravity (superprecipitation). Cytochalasin D had no effect on the sedimentation pattern, while bistheonellide A, swinholide A, and mycalolide B increased the amount of actin left in supernatant (Fig. 2B). These results also suggest that the bistheonellide A-actin complex is different in conformation from the cytochalasin D-actin complex or native F-actin. We have reported that mycalolide B inhibited superprecipitation of native actomyosin from chicken gizzard muscle (*27*) and activation by actin of myosin ATPase activity of rabbit skeletal muscle (*4*). On the other hand, cytochalasin D did not inhibit superprecipitation (*28*) or actin activation of myosin ATPase (*4*). From these results, it is postulated that bistheonellide A binds not to actin dimer but to two G-actin molecules symmetrically through its two putative actin-binding sites (Fig. 1). The fact that mycalolide B (*4*) and aplyronine A (*12*) bind one G-actin molecule while swinholide A (*13*) and bistheonellide A bind two G-actin molecules supports the hypothesis that the side chains rather than the macrolide rings in these macrolides account for the actin-binding property (*5*).

The depolymerization of F-actin by bistheonellide A was slow (half time$\gg$1 min; see Fig. 4A). A slow depolymerization rate was also observed with the actin-capping agent cytochalasin D (half time$\gg$1 min), while that with swinholide A was fast (half time $<$1 min). The actin-severing macrolides mycalolide B and aplyronine A also rapidly depolymerize F-actin (*4, 12*). Although bistheonellide A and swinholide A had similar effects on the steady levels of actin polymerization, their effects on the depolymerization rate were different. This difference may be due to their different severing activities. Actin polymerization consists of three phases: nucleation, elongation, and steady state (*25, 29*). In the elongation phase, in which G-actin binds to F-actin (*i.e.*, the nucleus), swinholide A-treated actin polymerized faster than bistheonellide A-treated actin (Fig. 4B). This difference may have resulted from an increased number of nuclei after treatment with swinholide A, since mycalolide B (*4*), aplyronine A (*12*), and nibbling proteins (*6-8*) increase the number of nuclei and consequently increase the velocity of actin polymerization. As shown in Fig. 5, mycalolide B and swinholide A shortened the half-time, while bistheonellide A showed almost no effect. This result suggests that the different effects on the rate of F-actin depolymerization between bistheonellide A and swinholide A (see Fig. 4A), as well as the difference in their inhibitory effects on actin polymerization (Fig. 4B), are due to a difference in their severing activities.

Bistheonellide A depolymerized F-actin in linear manner, showing the half-maximal effect at a molar ratio of bistheonellide A to actin of approximately 0.25. However, no severing effect was observed as reported previously (*30*), even at a molar ratio of more than 1. Although mycalolide B also depolymerized F-actin in linear manner (*4*), its severing effect was not linear, the half-maximal effect being observed at the molar ratio of mycalolide B to actin of approximately 0.01 (Fig. 5B). On the other hand, the F-actin capping agent cytochalasin D maximally inhibited actin polymerization at substoichiometric concentrations (*4*). This is probably because one molecule of capping agent is sufficient for one F-actin molecule to depolymerize, although one F-actin molecule contains several hundred actin molecules (*31, 32*). Like the capping agent, it seems that one severing agent is sufficient to sever one F-actin, as mycalolide B demonstrated maximal severing effect at substoichiometric concentrations (Fig. 5B). Compared with mycalolide B, swinholide A has approximately 100 times lower ability to sever F-actin (Fig. 5B). The absence or weakness of severing activity in bistheonellide A and swinholide A may be the result of either (i) the weakness of their binding (low affinity) to F-actin, or (ii) their low ability to induce conformational change of actin protomer to nibble it from F-actin. In the former case, these compounds would be displaced by a severing agent with higher affinity to F-actin if these two types of compounds shared the same binding site on actin. In the latter case, these compounds would inhibit the severing agent if they already occupied the common binding site on actin. As shown in Fig. 5C, the presence of bistheonellide A or swinholide A (at the same concentration as actin) had no inhibitory effect on the mycalolide B-induced increment of depolymerizing rate, *i.e.*, a one-quarter concentration of mycalolide B can easily overcome the action of bistheonellide A or swinholide A. Mycalolide B and bistheonellide A appear to share the same binding site on actin, since both can inhibit the binding of biocytinylated mycalolide B-analogue to G-actin (*33*). Therefore, this result suggests that bistheonellide A and swinholide A have lower affinity than mycalolide B to F-actin.

The F-actin capping agent cytochalasin D had little inhibitory effect on F-ADP-actin polymerization (Fig. 6A), since its effect depends on the difference in critical concentration between the two ends of F-actin (*21*). On the other hand, bistheonellide A depolymerizes F-ADP-actin, which suggests that it acts to depolymerize F-actin not *via* capping but by sequestering G-actin to prevent its interaction with F-actin. Figure 6B suggests that this macrolide-G-actin complex can never polymerize, but the possibility that these macrolides denatured actin to prevent polymerization cannot be excluded.

Bistheonellide A and swinholide A have identical side chains (Fig. 1), form tertiary complexes with two G-actin molecules, and sequester G-actin from polymerization.

However, swinholide A, but not bistheonellide A, has a severing effect, even though it is weak. Swinholide A decreased the rate of nucleotide exchange (Fig. 7B), as reported for mycalolide B and aplyronine A (*12*), while bistheonellide A increased it (Fig. 7A). Binding of these macrolides to G-actin may elicit different conformational changes in actin, and this may account for the different severing activities of these dimeric macrolides.

Bistheonellide A, swinholide A, mycalolide B, and aplyronine A have a common motif: C-19 to C-25 of bistheonellide A, C-21 to C-27 of swinholide A, C-24 to C-30 of mycalolide B, C-23 to C-29 of aplyronine A, and an ester structure involving C-1 of the macrolide ring (Fig. 1). These common structures may be necessary for binding to G-actin but not for severing F-actin. Bistheonellide A has the same structure as swinholide A except for the lack of C-2 and C-3. Therefore, the absence of these two carbons may account for attenuation of severing activity for F-actin. By deletion of these carbons, the methyl group at C-4 of swinholide A is shifted to C-2 in bistheonellide A. Since other macrolides with severing effects do not have a methyl group in this position, this methyl group in bistheonellide A may abolish the severing activity. The weaker severing activity of swinholide A compared with mycalolide B and aplyronine A may be due to the tetrahydropyran ring, which is open and contains a few carbons between *N*-methyl-formylamide in mycalolide B or aplyronine A.

In conclusion, bistheonellide A inhibited actin polymerization by forming a complex with G-actin. This effect is similar to those of mycalolide B, aplyronine A, and swinholide A. Bistheonellide A has two copies of the actin-binding motif common to mycalolide B and aplyronine A, and it bound to actin in a molar ratio of 1:2. Its severing effect on F-actin was weakest among these four macrolides. These results suggest that bistheonellide A, mycalolide B (*4*), aplyronine A (*12*), swinholide A (*13*), and tolytoxin (*34*) compose a family of "actin-depolymerizing macrolides." The side chain common to these macrolides may account for the actin-binding activity.

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