J. Biochem. 120, 552-555 (1996)

Novel Actin Depolymerizing Macrolide Aplyronine A[1]

Shin-ya Saito,*,[2] Shugo Watabe,† Hiroshi Ozaki,* Hideo Kigoshi,‡ Kiyoyuki Yamada,‡ Nobuhiro Fusetani,§ and Hideaki Karaki*

Department of Veterinary Pharmacology, †Laboratory of Aquatic Biotechnology, and §Laboratory of Marine Biochemistry, Graduate School of Agriculture and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113; and ‡Department of Chemistry, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464

Received for publication, April 4, 1996

Aplyronine A is a macrolide isolated from *Aplysia kurodai*. By monitoring fluorescent intensity of pyrenyl-actin, it was found that aplyronine A inhibited both the velocity and the degree of actin polymerization. Aplyronine A also quickly depolymerized F-actin. The kinetics of depolymerization suggest that aplyronine A severs F-actin. The relationship between the concentration of total actin and F-actin at different concentrations of aplyronine A suggests that aplyronine A forms a 1:1 complex with G-actin. From these results, it is concluded that aplyronine A inhibits actin polymerization and depolymerizes F-actin by nibbling. Comparison of the chemical structure of aplyronine A and another actin-depolymerizing macrolide, mycalolide B, suggests that the side-chain but not the macrolide ring of aplyronine A may account for its actin binding and severing activity.

Key words: actin, actin depolymerization, aplyronine A, macrolide, mycalolide B.

Aplyronine A is a macrolide isolated from the sea hare *Aplysia kurodai* from the Pacific coast of Mie Prefecture, Japan (*1*). Previously, it has been shown that a macrocyclic trisoxazole, mycalolide B (*2*), depolymerizes F-actin (*3*), and inhibits smooth muscle contraction (*4*). Because aplyronine A and mycalolide B have different macrolide rings and similar side-chains (Fig. 1), comparison of the biochemical effects of these two compounds may clarify the structure-activity relationship. For this purpose, we examined the effects of aplyronine A on actin. Results indicated that aplyronine A has the actin-depolymerizing effect, suggesting that the side-chain, but not the macrolide ring, binds to actin to depolymerize the filament.

MATERIAL AND METHODS

Preparation of Proteins—Actin was purified from rabbit skeletal muscle (*5*) using buffer G containing 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, and 2 mM Tris (pH 8.0 with HCl) as described previously (*3*). The crude G-actin was subjected to Sephadex G-200 gel filtration. Undenatured G-actin fraction was collected and used. Tropomyosin was purified from rabbit skeletal muscle (*6*) and its concentration was determined by measuring absorbance at 276 nm using an extinction coefficient of 24,500 $M^{-1} \cdot cm^{-1}$ (*7*).

Actin Labeling and Measurement of Fluorescence of Pyrenyl-Actin—Pyrene labeling was performed by the method described previously (*3*). Actin polymerization was started by the addition of 50 mM KCl and 2 mM $MgCl_2$ to buffer G. The time course of polymerization (or depolymerization) was continuously monitored by measuring fluorescence of pyrenyl-actin (2.5% of total actin) with a fluorometer (FP-2060, Japan Spectroscopic) at 25°C at 365 nm excitation and 407 nm emission wavelengths.

Nucleotide Exchange Assay—The G-actin solution was diluted 100 times with a buffer containing 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 2 mM Tris (pH 8.0 with HCl), 10 min before the addition of 50 μM εATP. The fluorescence of εATP was monitored with the FP-2060 fluorometer at 360 nm excitation and 410 nm emission wavelengths at 25°C (*8*).

Chemicals—Aplyronine A was isolated from sea hare *Aplysia kurodai* by the method described previously (*1*). Mycalolide B was isolated from marine sponge *Mycale* sp. by the method described previously (*2*). *N*-(1-Pyrene)-iodoacetamide and 1,N^6-ethenoadenosine 5′ triphosphate (εATP) were obtained from Molecular Probes (Eugene, OR).

RESULTS AND DISCUSSION

Addition of 50 mM K^+ and 2 mM Mg^{2+} polymerized G-actin after a lag phase (Fig. 2). Aplyronine A inhibited both the velocity and the degree of polymerization without changing the lag phase (Fig. 2). Figure 3A shows that aplyronine A depolymerized F-actin. Aplyronine A accelerated the depolymerization of F-actin in two phases, a rapid first phase (<1 min) and a slow second phase (>1 min). To determine whether aplyronine A has severing activity, we compared the number of F-actin before and after treatment with aplyronine A by a dilution method. In this method, the decay of fluorescence after dilution of F-actin is considered to be due to depolymerization from both ends of F-actin,

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.
[2] To whom correspondence should be addressed at the present address: Department of Veterinary Pharmacology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka 020. Phone: +81-196-21-6214, Fax: +81-196-21-6215
Abbreviations: pyrenyl-actin, pyrene-labeled actin; εATP, 1,N^6-ethenoadenosine 5′ triphosphate.

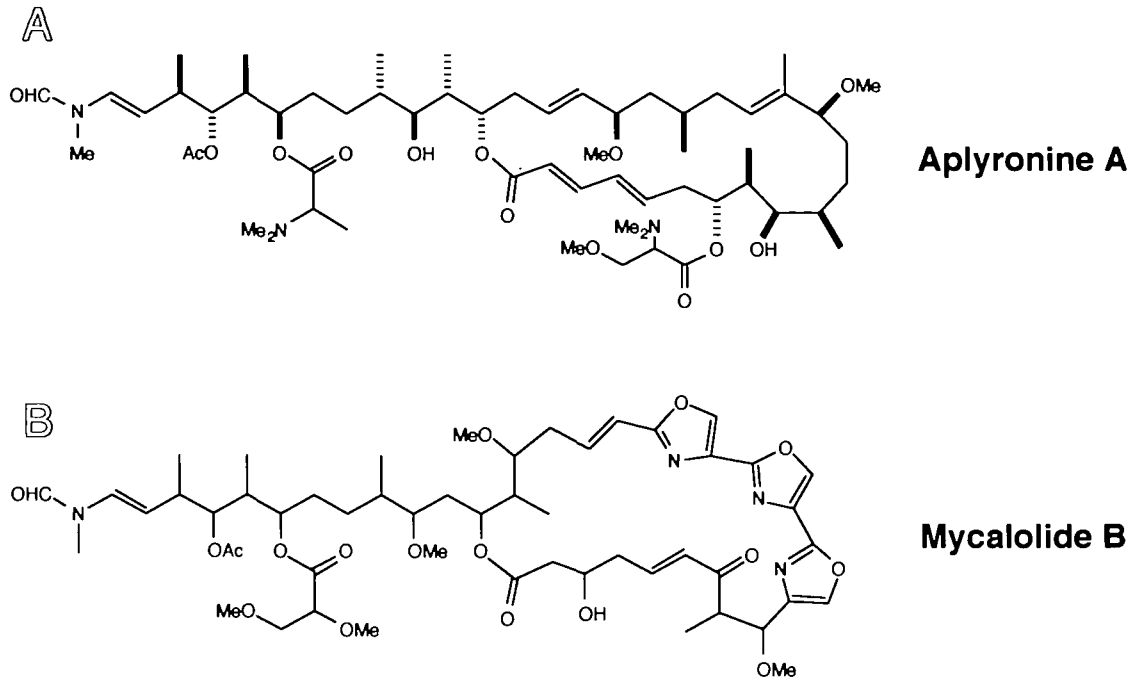

Fig. 1. **Chemical structures of aplyronine A (A) and mycalolide B (B).** Note that compounds have different macrolide rings and similar side-chains.

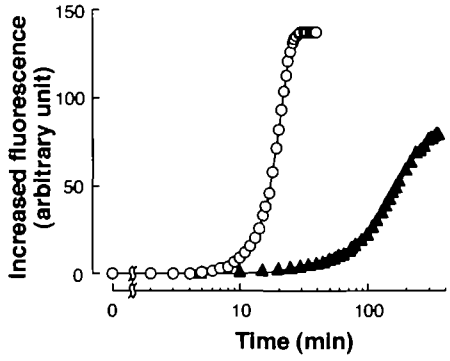

Fig. 2. **Time course of the inhibitory effect of aplyronine A on actin polymerization.** Actin (4 μM) was treated without (○) or with 1 μM aplyronine A (▲) for 12 min before starting the polymerization at 25°C. At time 0, 50 mM KCl and 2 mM $MgCl_2$ were added and polymerization was monitored with fluorometer.

and thus the rate of depolymerization should be proportional to the number of F-actin (*3*). When aplyronine A was added to F-actin simultaneously with dilution, the initial rate of depolymerization was markedly increased as shown in Fig. 3B. This result suggests that aplyronine A severs the F-actin.

When F-actin was pretreated with aplyronine A for 1.5 min, the intensity of fluorescence was decreased by approximately 50%. Subsequently, the actin solution was diluted and the rate of decrease in fluorescence was monitored. Aplyronine A accelerated the depolymerization induced by dilution, decreasing $t_{1/2}$ from 0.8 to 0.3 min. When F-actin was treated with aplyronine A for 17.5 or 30 min, the fluorescent intensity before dilution was the same as that after a 1.5-min treatment. However, the rate of depolymerization delayed again after a longer treatment with aplyronine A ($t_{1/2}$ was 0.5 min for 17.5 or 30-min treatment) (Fig. 3B). These results suggest that after aplyronine A has severed F-actin, aplyronine A/G-actin complex may be sequestered and fragmented short F-actin may be reannealed to form longer filaments by binding in an end-to-end manner as described by Wegner (*9*).

Figure 4 shows the relationship between the fluorescence of F-actin and the total actin concentration in the absence or presence of aplyronine A. In the absence of aplyronine A, fluorescence increased linearly as a function of actin concentration. Aplyronine A (1 and 2 μM) shifted the concentration-fluorescence relationship to the right in parallel, indicating that aplyronine A only increased critical concentration, and had no other effect on the concentration-fluorescence relationship. This result suggests that aplyronine A sequesters G-actin at a steady state. The K_d of aplyronine A to G-actin was calculated to be 0.10 μM assuming that aplyronine A binds G-actin in a 1:1 ratio.

It has been shown that depactin, a monomer-sequestering protein with severing activity, increased the rate of actin polymerization (elongation) by increasing the number of F-actin without facilitating nucleation (*10*). On the other hand, profilin, a monomer-sequestering protein without severing activity (*11*), affected neither nucleation nor elongation. Although aplyronine A seems to have both severing (Fig. 3) and sequestering (Fig. 4) activities, as depactin does, it changed neither nucleation nor elongation as did profilin (Fig. 2). This discrepancy suggests that the binding affinity of aplyronine A to G-actin may be stronger than that to F-actin and, therefore, the effective concentration of aplyronine A to sever F-actin becomes low when the concentration of G-actin is increased. Mycalolide B, which has both severing and sequestering activities, also did not change nucleation and elongation. The effect of mycalolide B may be explained by a similar mechanism (*3*). Further

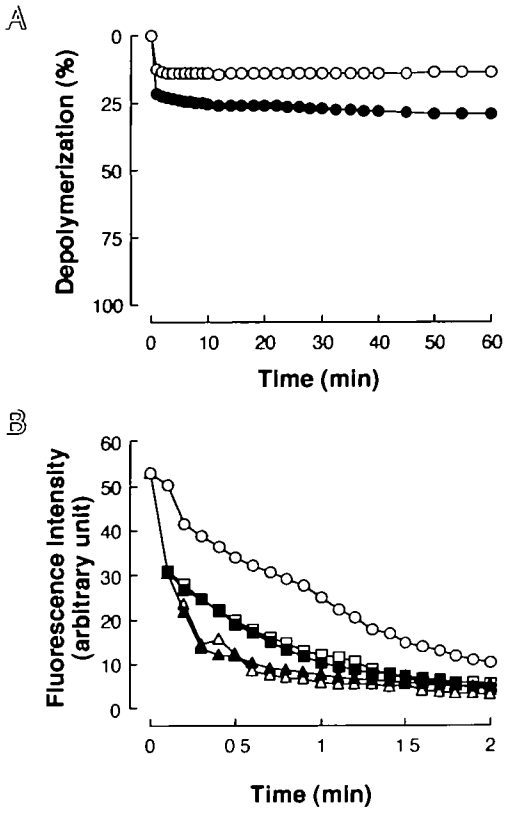

Fig. 3. **A: Time course of the depolymerizing effect of aplyronine A on F-actin.** Aplyronine A was applied to F-actin at 0 min and the decrease in fluorescence intensity was monitored. 100%: fluorescence intensity of G-actin (4 μM) before polymerization. 0%: fluorescence intensity of F-actin before the application of aplyronine A (○, 0.5 μM; ●, 1 μM). **B: Spontaneous depolymerization by dilution.** Actin (12 μM) was polymerized with 50 mM KCl and 2 mM $MgCl_2$ and then diluted by 160 times at time 0. ○: control (final actin concentration = 75 nM). ▲: diluted with aplyronine A (37.5 nM). F-actin was treated with aplyronine A (6 μM) for 1.5 min (△), 17.5 min (■) or 30 min (□) and then diluted with buffer to final concentration of 75 nM actin and 37.5 nM aplyronine A.

studies are necessary to examine this possibility.

Phalloidin is known to bind F-actin in stoichiometry with each actin protomer and to inhibit both polymerization and depolymerization (*12*). As shown in Fig. 5, pretreatment of F-actin (2.5 μM) with phalloidin (2.5 μM) completely inhibited the depolymerizing effect of aplyronine A. In contrast, tropomyosin (0.5 μM), which is also known to stabilize F-actin (*13, 14*), had no effect on the aplyronine A-induced depolymerization. Our unpublished observation showed that aplyronine A inhibited smooth muscle contraction in which actin filament is coated with tropomyosin. From these results, it is suggested that tropomyosin may not attenuate the action of aplyronine A.

G-Actin binds ATP in a 1:1 molar ratio, and this ATP is exchangeable with free ATP. One can monitor the rate of ATP exchange using a fluorescent analogue of ATP, εATP (*8*). It has been reported that the G-actin-binding proteins either increase (*15*) or decrease (*16-18*) the exchange rate. As shown in Fig. 6, aplyronine A inhibited the rate of ATP exchange in a concentration-dependent manner. This result suggests that aplyronine A causes a conformational change

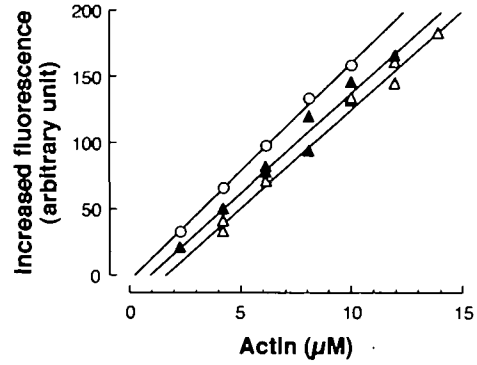

Fig. 4. **Effects of aplyronine A on fluorescence intensity of actin.** The fluorescence intensity of actin incorporated into filaments was plotted as a function of the total actin concentration in the presence of 0, 1, and 2 μM aplyronine A. F-Actin was depolymerized for 120 min in the absence (○) or presence of 1 μM (▲) or 2 μM (△) aplyronine A. Difference between the fluorescent intensity of G-actin and that of F-actin is plotted on the ordinate. The intersection with the x-axis in the presence of 0, 1, and 2 μM aplyronine A was 0.24, 0.95, and 1.64 μM, respectively, suggesting a K_d of 0.10 μM if one molecule of aplyronine A binds one molecule of actin.

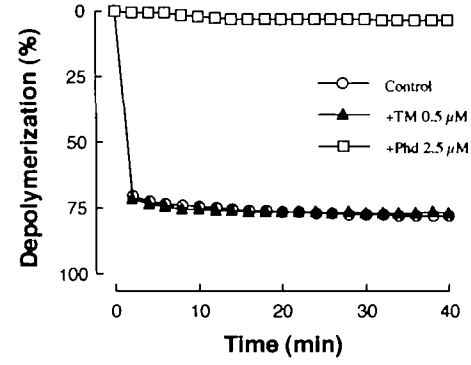

Fig. 5. **Effect of phalloidin and tropomyosin on aplyronine A-induced depolymerization.** Experimental conditions are the same as those in Fig. 3. Aplyronine A (2 μM) was applied to F-actin (2.5 μM) in the absence (○) or presence of 2.5 μM phalloidin (□) or 0.5 μM tropomyosin (▲).

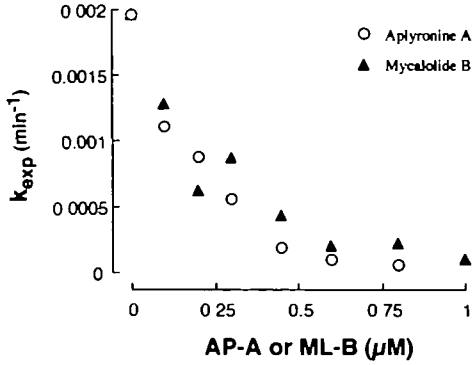

Fig. 6. **Effect of aplyronine A and mycalolide B on the exchange rate of εATP with ATP bound to G-actin.** Relationship between the observed binding coefficient (k_{exp}) and concentration of aplyronine A (○) or mycalolide B (▲) is shown.

in the G-actin molecule.

In conclusion, aplyronine A binds to G-actin in a 1:1 molecular ratio, sequesters G-actin from polymerization and also severs F-actin. Previously, we have shown that a marine toxin, mycalolide B, isolated from marine sponge, inhibited the velocity and the degree of actin polymerization. Mycalolide B inhibited polymerization by sequestering G-actin and depolymerized F-actin by severing activity (*3*). It has also been shown that mycalolide B-induced depolymerization was inhibited by phalloidin (*3*) but not by tropomyosin (unpublished observation). These results are similar to those of aplyronine A. Comparing the chemical structures of aplyronine A and mycalolide B (Fig. 1) suggests that the side-chain, but not macrolide ring, may play an important role in the actin-inhibiting activity of the actin-depolymerizing macrolides.

REFERENCES

1. Yamada, K., Ojika, M., Ishigaki, T., and Yoshida, Y. (1993) Aplyronine A, a potent antitumor substance, and the congeners aplyronines B and C isolated from the sea hare *Aplysia kurodai*. *J. Am. Chem. Soc.* **115**, 11020-11021
2. Fusetani, N., Yasumuro, K., Matsunaga, S., and Hashimoto, K. (1989) Mycalolides A-C, hybrid macrolides of ulapualides and halichondramide, from a sponge of the genus *Mycale*. *Tetrahedron Lett.* **30**, 2809-2812
3. Saito, S., Watabe, S., Ozaki, H., Fusetani, N., and Karaki, H. (1994) Mycalolide B, a novel actin depolymerizing agent. *J. Biol. Chem.* **269**, 29710-29714
4. Hori, M., Saito, S., Shin, Y.Z., Ozaki, H., Fusetani, N., and Karaki, H. (1993) Mycalolide-B, a novel and specific inhibitor of actomyosin ATPase isolated from marine sponge. *FEBS Lett.* **322**, 151-154
5. Spudich, J.A. and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**, 4866-4871
6. Bailey, K. (1948) Tropomyosin: A new asymmetric protein component of the muscle fibril. *Biochem. J.* **43**, 271-279
7. Wegner, A. (1979) Equilibrium of the actin-tropomyosin interaction. *J. Mol. Biol.* **131**, 839-853
8. Waechter, F. and Engel, J. (1975) The kinetics of the exchange of G-actin-bound 1:N^6-ethenoadenosine 5′-triphosphate with ATP as followed by fluorescence. *Eur. J. Biochem.* **57**, 453-459
9. Wegner, A. (1982) Spontaneous fragmentation of actin filaments in physiological conditions. *Nature* **296**, 266-267
10. Mabuchi, I. (1983) An actin-depolymerizing protein (depactin) from starfish oocytes: Properties and interaction with actin. *J. Cell Biol.* **97**, 1612-1621
11. Tobacman, L.S. and Korn, E.D. (1981) The regulation of actin polymerization and the inhibition of monomeric actin ATPase activity by *Acanthamoeba* profilin. *J. Biol. Chem.* **257**, 4166-4170
12. Sampath, P. and Pollard, T.D. (1992) Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. *Biochemistry* **30**, 1973-1980
13. Wegner, A. (1982) Kinetic analysis of actin assembly suggests that tropomyosin inhibits spontaneous fragmentation of actin filaments *J. Mol. Biol.* **161**, 217-227
14. Ishikawa, R., Yamashiro, S., and Matsumura, F. (1989) Annealing of gelsolin-severed actin fragments by tropomyosin in the presence of Ca^{2+}. Potentiation of the annealing process by caldesmon. *J. Biol. Chem.* **264**, 16764-16770
15. Mockrin, S.C. and Korn, E.D. (1980) *Acanthamoeba* profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine 5′-triphosphate. *Biochemistry* **19**, 5359-5362
16. Mannherz, H.G., Goody, R.S., Konrad, M., and Nowak, E. (1980) The interaction of bovine pancreatic deoxyribonuclease I and skeletal muscle actin. *Eur. J. Biochem.* **104**, 367-379
17. Hitchcock, S.E. (1980) Actin deoxyroboncuclease I interaction. Depolymerization and nucleotide exchange. *J. Biol. Chem.* **255**, 5668-5673
18. Nishida, E. (1985) Opposite effects of cofilin and profilin from porcine brain on rate of exchange of actin-bound adenosine 5′-triphosphate. *Biochemistry* **24**, 1160-1164