Purealin, a Novel Stabilizer of Smooth Muscle Myosin Filaments that Modulates ATPase Activity of Dephosphorylated Myosin*

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Effects of purealin isolated from a sea sponge, Psammaplysilla purea, on the enzymatic and physicochemical properties of chicken gizzard myosin were studied. At 0.15 M KCl, 40 μM purealin increased the Ca2+- and Mg2+-ATPase activity of dephosphorylated gizzard myosin to 2.5- and 3-fold, respectively, but decreased the K'+EDTA-ATPase activity of the myosin to 0.25-fold. In contrast, purealin had little effect on the ATPase activities of phosphorylated gizzard myosin. The ATP-induced decrease in light scattering of dephosphorylated gizzard myosin at 0.15 M KCl was lessened by 40 μM purealin. Electron microscopic observations indicated that thick filaments of dephosphorylated myosin were disassembled immediately by addition of 1 mM ATP at 0.15 M KCl, although they were preserved by purealin for a long time even after addition of ATP. Upon ultracentrifugation, dephosphorylated myosin sedimented as two components, the 10 S species and myosin filaments, in the solution containing 0.18 M KCl and 1 mM Mg-ATP in the presence of 60 μM purealin. These results suggest that purealin modulates the ATPase activities of dephosphorylated gizzard myosin by enhancing the stability of myosin filaments against the disassembling action of ATP.

Phosphorylation of the 20,000-Da light chain of smooth muscle myosin is one of the primary factors for the maintenance of myosin filaments in Mg-ATP in vitro. Thick filaments of dephosphorylated gizzard myosin were disassembled by addition of stoichiometric amounts of ATP at 0.15 M KCl, whereas the filaments of phosphorylated myosin remained assembled (1-3). The disassembled monomer myosin sedimented at 10 S on ultracentrifugation (2, 4) and formed a looped or folded structure in which the tail of the myosin molecule was bent back toward the head region (4-6). At higher ionic strength, gizzard myosin sedimented at 6 S and had an extended tail. The correlation between conformation and ATPase activity of the myosin was already reported (1, 7). Furthermore, the possible involvement of a conformational transition of myosin in regulation of activity in smooth muscle actomyosin has been suggested (7). Hence, to understand the correlation, smooth muscle researches have been looking for the novel tool that provides some information on the determinants of conformational changes among 10 S, 6 S, and filamentous form of myosin.

In the course of our survey of bioactive compounds in marine organisms, purealin (Fig. 1) was isolated from an Okinawa sea sponge, Psammaplysilla purea (8). We have already found that purealin activates the K'+EDTA-ATPase activity of myosin from rabbit skeletal muscle and superprecipitation of myosin B (9). In this paper, we have shown for the first time that purealin enhances the stability of thick filaments of dephosphorylated gizzard myosin against the disassembling action of ATP.

MATERIALS AND METHODS
Purealin was isolated from an Okinawa sea sponge, P. purea. Details of the purification have been published (8). Purealin was dissolved in dimethyl sulfoxide, and a final concentration of dimethyl sulfoxide did not exceed 1%. Less than 1% dimethyl sulfoxide had little effect on the ATPase activities of gizzard myosin.

Proteins were prepared by the following procedures. Myosin was prepared from fresh chicken gizzard as described by Ebsahi (10) with a slight modification (11). Myosin light chain kinase was obtained from fresh chicken gizzard by the method of Adelstein and Klee (12) with a slight modification (11). Calmodulin was purified from frozen bovine brain (11). HMM 7 was prepared by chymotryptic hydrolysis of dephosphorylated gizzard myosin according to Onishi and Watanabe (13) with slight modifications. Chymotryptic digestion was carried out by incubating gizzard myosin (20 mg/ml) with 0.05 mg/ml a-chymotrypsin at 20 °C for 6 min in 0.4 M KCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol. The digestion was stopped by adding 0.1 mM diisopropyl fluorophosphate (Sigma). The reaction mixture was added with 3 volumes of cold 13.3 mM MgCl2 and stood for 1 h on ice, and then the mixture was centrifuged at 80,000 × g for 30 min. The supernatant was used as gizzard HMM and the precipitate was used as source of LMM. LMM was obtained from the precipitate mentioned above, followed by alcohol denaturation and extraction in a high-salt buffer (14). Stable phosphorylated gizzard myosin was obtained according to Nakamura and Nonomura (11) as follows. Dephosphorylated myosin was incubated in a solution containing 50 mM KCl, 8 mM MgCl2, 0.1 mM CaCl2, 20 mM Tris-HCl (pH 7.5), 2 mM ATP, 10 μg/ml myosin light chain kinase, and 2 μg/ml calmodulin at 25 °C for 15 min. KCl and potassium phosphate (pH 7.2) were then added to the reaction mixture to a final concentration of 400 mM and 10 mM, respectively. Immediately, the mixture was applied to a hydroxylapatite column equilibrated with 400 mM KCl and 10 mM potassium phosphate (pH 7.2) and eluted with the same buffer. The passed-through fraction was used as stable phosphorylated gizzard myosin. The levels of light chain phosphorylation of dephosphorylated and phosphorylated myosin used in this study were below 5% and above 95%, respectively. The purity of proteins was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli (15). The levels of phosphorylation of the 20,000-Da light chain of gizzard myosin were assessed by urea.

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The abbreviations used are: HMM, heavy meromyosin; LMM, light meromyosin; Mops, 3-(N-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.
glycerol gel electrophoresis according to Perrie and Perry (16). α-Chymotrypsin (type II) was purchased from Sigma. All other reagents used were of analytical grade.

ATPase activities were determined by measuring the amount of P released according to Martin and Doty (17). A reaction mixture (1 ml) containing gizzard myosin or HMM with purealin was preincubated for 5 min. The ATPase reaction was started by adding ATP and stopped by adding 0.5 ml of 20% trichloroacetic acid. Assay conditions are given in figure legends. Less than 20% of the substrate was hydrolyzed in all assays. Data on ATPase presented in figures are representative of 2–5 experiments. The light-scattering intensity at an angle of 90° was recorded by using a spectrofluorometer (Aminco SPF-500™) with a temperature-controlled cell holder and a magnetic stirrer at 29°C. Sedimentation velocity measurements were carried out in an analytical ultracentrifuge (Hitachi UCA-1A) at 13°C, using the schlieren optical system. The partial specific volume of gizzard myosin at 20°C was assumed to be equal to that of skeletal myosin (0.729 ml/g) (18). Electron microscopic observations of gizzard myosin and LMM were carried out using the negative-staining technique (carbon-coated collodion grids, stained with uranyl acetate). A JEM-1200EX electron microscope was used at 80 kV.

RESULTS

Effects of Purealin on the Ca2+-, K+-EDTA- and Mg2+-ATPase Activity of Myosin—The KCl dependence of ATPase activity of dephosphorylated myosin differed greatly from that of phosphorylated myosin. The Ca2+-ATPase activity of phosphorylated myosin slightly increased with a decrease in the KCl concentrations from 0.5 to 0.1 M (Fig. 2A). In the Ca2+ and Mg2+-ATPase activities of dephosphorylated myosin, depression of the activity was observed around 0.2 M KCl (Fig. 2, A and C). The K+-EDTA-ATPase activity of phosphorylated myosin decreased monotonously with a decrease in the KCl concentrations. In the activity of dephosphorylated myosin, a marked enhancement was observed around 0.2 M KCl (Fig. 2B). These characteristics of KCl dependence of dephosphorylated myosin ATPases were in good agreement with those in the previous reports of Onishi et al. (1) and Ikebe et al. (7).

FIG. 1. Chemical structure of purealin.

FIG. 2. Effects of purealin on the Ca2+ (A), K+-EDTA- (B), and Mg2+-ATPase (C) activity of phosphorylated and dephosphorylated gizzard myosin in the presence of various concentrations of KCl. The ATPase reaction was started by adding 1 mM ATP after 5 min preincubation of gizzard myosin with purealin in the reaction medium. The preparation of stable phosphorylated gizzard myosin was described under "Materials and Methods." Assay conditions: A, 10 mM CaCl2, 20 mM Mops (pH 6.8), 1 mM ATP, 0.077 mg/ml phosphorylated (C), or 0.1 mg/ml dephosphorylated (C) myosin, and 20 μM purealin (●, ▲) or 1% dimethyl sulfoxide (O, □) at 30°C. B, 0 mM EDTA, 20 mM Mops (pH 6.8), 1 mM ATP, 0.1 mg/ml phosphorylated (●, ▲) or dephosphorylated (O, □) myosin, and 20 μM purealin (●, ▲) or 1% dimethyl sulfoxide (O, □) at 30°C. C, 10 mM MgCl2, 0.1 mg/ml phosphorylated (●, ▲) or 1% dimethyl sulfoxide (O, □) at 30°C. Data on ATPase presented in figures are representative of 2–5 experiments.
Effects of purealin on the sedimentation coefficient of dephosphorylated gizzard myosin

Ultraacentrifugation of dephosphorylated gizzard myosin (1 mg/ml) was performed at 13°C in the solution containing 0.18 M KCl, 10 mM MgCl₂, 1 mM EGTA, and 40 mM Mops (pH 6.8) in the presence or absence of 1 mM ATP. Rotor speed, 61,200 and 6,950 rpm. Sedimentation coefficients ($S_{20,w}$) were obtained from the schlieren patterns in the sedimentation studies.

<table>
<thead>
<tr>
<th>ATP</th>
<th>Control</th>
<th>60 μM purealin</th>
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<tbody>
<tr>
<td>None</td>
<td>135</td>
<td>132</td>
</tr>
<tr>
<td>1 mM</td>
<td>10.0</td>
<td>10.3</td>
</tr>
<tr>
<td>60 μM</td>
<td>136</td>
<td></td>
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ATP-induced decrease of light scattering was lessened by 40 μM purealin (Fig. 4). The light-scattering intensity was not decreased completely by ATP even at the concentration of 0.1 mM (ATP/myosin = 167).

The time course of the ATP-induced decrease of light-scattering intensity is shown in the inset of Fig. 4. The light-scattering intensity fully decreased within 3 min ($t_0$ was within 0.2 min) after addition of ATP at all concentrations tested in control experiments (Fig. 4, inset, a). However, in the solution containing 40 μM purealin, light-scattering intensity decreased slowly ($t_0$ was 3 min) after addition of 0.1 mM ATP and reached the minimum value at 35 min (Fig. 4, inset, b).

Although light-scattering experiments suggested that purealin might favor thick filaments over myosin monomers after addition of ATP, it was desirable to confirm this possibility directly using an electron microscope. Electron micrographs of dephosphorylated myosin at 0.15 M KCl (Fig. 5, A and B) indicate that thick filaments were readily dissociated by addition of 1 mM ATP as already described (1–3). In contrast, appreciable thick filaments were observed in the presence of 60 μM purealin even more than 15 min after addition of 1 mM ATP (Fig. 5, C and D). The results shown in these micrographs were consistent with the light-scattering data.

Effects of purealin on the sedimentation coefficients of dephosphorylated myosin at 0.18 M KCl are shown in Table I. Addition of 1 mM ATP to dephosphorylated myosin filaments depolymerized the polymer into a 10 S component, as reported by Suzuki et al. (2) and Trybus et al. (4). In the presence of 60 μM purealin, two sedimenting components, the...
Fig. 5. Electron micrographs of negatively stained thick filaments of dephosphorylated gizzard myosin with or without purealin. Thick filaments of dephosphorylated gizzard myosin (final concentration of 0.3 mg/ml) were formed at room temperature in the solution containing 0.15 M KCl, 10 mM MgCl₂, 1 mM EGTA, 20 mM imidazole (pH 6.8) in the presence of 0.6% dimethyl sulfoxide. A, control; B, 15 min after addition of 1 mM ATP to the control solution; C, in the presence of 60 μM purealin; D, 15 min after addition of 1 mM ATP to the solution with purealin. Samples were negatively stained with 4% uranyl acetate. An inserted bar indicates 500 nm.

Fig. 6. Effects of purealin on sedimentation patterns of dephosphorylated gizzard myosin. Ultracentrifugation of dephosphorylated myosin (1 mg/ml) was performed in the solution containing 1 mM ATP, 0.18 M KCl, 10 mM MgCl₂, 1 mM EGTA, and 40 mM Mops (pH 6.8) in the presence or absence of 60 μM purealin at 13 °C. A, patterns of dephosphorylated myosin in the presence of 60 μM purealin (upper) and 0.6% dimethyl sulfoxide (lower) at 19 min after reaching a speed of 8,380 rpm; bar angle 65°. An arrow indicates the peak corresponding to myosin filaments. B, patterns of dephosphorylated myosin in the presence of 60 μM purealin (upper) and 0.6% dimethyl sulfoxide (lower) at 37 min after reaching a speed of 51,200 rpm; bar angle 70°.

10 S species and myosin filaments were detected after addition of ATP (Fig. 6).

It is generally accepted that dephosphorylated myosin is 10 S monomers in the presence of ATP at 0.15 M KCl, whereas under the same condition phosphorylated myosin forms thick filaments, which are thought to be composed of 6 S monomers (1–3). In the present experiments, thick filaments of dephosphorylated myosin remained assembled in the presence of purealin under the same condition as described above (see Fig. 5). However, monomers dissociated from the filaments were unexpectedly sedimented at 10 S.

Effects of Purealin on the Subfragment of Dephosphorylated Myosin—HMM and LMM fragments were prepared from dephosphorylated myosin. The Ca²⁺-ATPase activity of HMM was decreased only slightly (about 0.2-fold) by 30 μM purealin in the range of KCl concentrations of 0.05–0.5 M (Fig. 7A). Depression of the activity seen in dephosphorylated myosin at 0.2 M KCl (Fig. 2A) was less prominent in dephosphorylated HMM (confirming the results of Onishi and Watanabe (13)). Purealin activates the Ca²⁺-ATPase activity of dephosphorylated myosin (Fig. 3A) but had no or little effect on the activity of dephosphorylated HMM (Fig. 7B). Effects of purealin on the Mg²⁺-ATPase activity of dephosphorylated HMM were also examined in the solution containing 0.15 M KCl, 10 mM MgCl₂, 40 mM Mops (pH 6.8), 1 mM ATP, 0.88 mg/ml gizzard HMM at 30 °C. Purealin caused no change in
the activities (data not shown).

Actin monomers, effects of purealin on LMM subfragments have not been detected in the presence or absence of ATP by light-scattering measurements (data not shown). Electron micrographs showed the presence of LMM filaments in the solution containing 0.15 M KCl with or without 10 mM MgCl₂. These filaments were not dissociated by addition of 1 mM ATP, as already reported by Scholey et al. (3).

**DISCUSSION**

Effects of purealin on the ATPase activities of dephosphorylated myosin in smooth muscle were most prominent at 0.15 M KCl (Fig. 2). It is well known that at this physiological ionic strength, thick filaments of dephosphorylated myosin are disassembled by stoichiometric addition of ATP into monomers having a sedimentation coefficient of 10 S (2–6). Results obtained from light-scattering measurements suggested that in the presence of purealin, thick filaments of dephosphorylated myosin still remained in filaments after addition of ATP (Fig. 4). This was confirmed by both electron microscopic observations and sedimentation velocity measurements (Figs 5 and 6). It was reported that the ATPase activity of myosin in a thick filament form was higher than that in an ATP dissociated form (1). On the basis of these observations, it is suggested that purealin activates the Ca²⁺ and Mg²⁺-ATPase activities of dephosphorylated myosin by enhancing the stability of thick filaments in the presence of Mg-ATP. However, the inhibition of K⁺-EDTA-ATPase activities by purealin could not be explained by this mechanism, because dephosphorylated myosin does not form thick filaments at 0.15 M KCl in the absence of MgCl₂ (20, 21).

Thick filaments of phosphorylated myosin remain assembled after addition of ATP (1–3). The changes in ATPase activities in myosin caused by purealin were in the same direction as expected from phosphorylation of the 20,000-Da light chain of myosin, that is, (1) the Ca²⁺- and Mg²⁺-ATPase activities were activated by purealin, whereas the K⁺-EDTA-ATPase activity was inhibited; (2) purealin modulated the ATPase activity of dephosphorylated myosin, but had no effect on that of phosphorylated myosin; and (3) the modulation by purealin occurred in the narrow range of KCl concentrations, 0.1–0.2 M. These results support our idea that purealin converts thick filaments of dephosphorylated myosin into filaments resistant to the disassembling action of ATP.

Effects of purealin on the ATPase activity of dephosphorylated HMM, which lost the ability to form filaments, were not detected in the present experiment. Moreover, purealin had little effect on LMM filaments in the light-scattering and electron microscopic experiments. These results provide further strong support of our view that purealin may modulate the ATPase activities of myosin through its stabilizing effect on thick filaments.

Stoichiometric amounts of ATP disassemble myosin filaments into 10 S monomers (1–6). It was suggested that ATP acted at a specific high-affinity binding site in the subfragment-1 portion of the myosin molecule, which might be the Mg²⁺-ATPase active site (3). Purealin modulated the activity of adenine nucleotidase, but not of phosphatases or lactate dehydrogenase. Therefore, it is probable that purealin acts on the ATP binding site of myosin.

It has been suggested that phosphorylation of myosin light chain is necessary for actin activation of the Mg²⁺-ATPase activity of myosin from smooth muscle (see review, Ref. 22). Therefore, phosphorylation-dependent changes in the conformation of myosin filaments may be directly responsible for the actin-myosin interaction in vivo. The changes in ATPase activities have been shown to correlate well with the thick filaments-monomer conversion (1), 10 S–6 S transition (7), and conformational changes in HMM (23, 24), but there are few reports on phosphorylation-dependent structural changes in myosin filaments (25). Thus, purealin may provide a useful tool to resolve the mechanism of filament assembly and disassembly of myosin and to elucidate the mechanism of conformational changes of myosin caused by phosphorylation, which in turn influence the actin-myosin interaction.

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**REFERENCES**