Articles

Purealin Blocks the Sliding Movement of Sea Urchin Flagellar Axonemes by Selective Inhibition of Half the ATPase Activity of Axonemal Dyneins†

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ABSTRACT: Ciliary and flagellar movements are explained by active sliding between the outer doublet microtubules of an axoneme via their inner and outer dynein arms. Purealin, a novel bioactive principle of a sea sponge *Psammaplysilla purea*, blocked the motility of Triton-demembranated sea urchin sperm flagella within 5 min at concentrations above 20 μM. In a similar concentration range, purealin blocked the sliding movement of the flagellar axonemes in vitro within a few minutes judging from the turbidity measurements. The ATPase activity of axonemes was partially inhibited by purealin in a concentration-dependent manner. The maximum inhibition reached approximately 50% at concentrations above 20 μM, indicating that half the axonemal ATPase activity is sensitive to purealin. Similar results were observed on the ATPase activity of outer-arm-depleted axonemes and that of a mixture of 21S dynein and salt-extracted axonemes. On the other hand, ATPase activity of isolated 21S dynein was not inhibited by purealin. The inhibitory action of purealin on the axonemal ATPases was reversed by dilution of purealin. The effect of purealin on the double-reciprocal plot of the ATPase activity as a function of ATP concentrations showed that the inhibition was not a competitive type. In accord with this finding, purealin did not affect the vanadate-mediated UV photocleavage of axonemal dyneins. These results suggest that purealin binds reversibly to a site other than the catalytic ATP-binding site and inhibits half the ATPase activity of axonemes. Taken together, our results suggest that purealin-sensitive ATPase activity of the dynein arms plays an essential role in generating the sliding movement of flagellar axonemes.

The bending waves of cilia and flagella are formed by the active sliding between the outer doublet microtubules of an axoneme via their inner and outer arms (*1*, *2*). The active components of the arms are known to be dynein ATPases, which convert chemical energy into mechanical energy to produce directed movement along microtubules toward their minus ends. Dynein molecules are large complex proteins (up to 1800 kDa) including two or three distinct heavy chains of about 500 kDa that have ATPase activity and several additional polypeptides called intermediate and light chains (*3*, *4*). The outer arms are constructed by homogeneous dynein molecules, which are arranged on the entire length of the outer doublet microtubules in a 24-nm periodicity (*5*). In contrast, arrangement of inner arms is more complicated. Three inner arms are arranged to form a triplet set, which repeats with a 96-nm periodicity along outer doublet microtubules in *Chlamydomonas* flagella (*6*, *7*).

There are several reports suggesting that inner and outer arms contribute in different ways to axonemal bending. In *Chlamydomonas* mutants lacking the outer arms, the flagella beat with a reduced beat frequency but with a normal waveform (*8*−*10*). In contrast, mutants lacking a large part of the inner arms do not beat at all. In other experiments, the extraction of outer arms from demembranated sea urchin sperm flagella results in a 50% reduction in the beat frequency, and it is restored to the normal beat frequency by adding back the outer dynein arms (*11*, *12*).

Dynein heavy chain has become central to the study of the function of dynein arms (*13*). In sea urchin sperm flagella, several heavy chains, which are designated as C, Aα, Aβ, D (doublets), and B (doublets) in order of increasing rate of migration on SDS−polyacrylamide gel electrophoresis (*14*), are considered to be components of the inner or outer arm dyneins. The outer arm dynein contains parts of Aα and Aβ heavy chains, respectively (*15*, *16*), and is selectively extracted by a high salt solution as a 21S particle (21S dynein). In contrast, there is little information on the heavy chain compositions and biochemical properties of each inner arm dynein. Recently, it has been suggested that one component of inner arm dyneins is composed of C and part of Aα heavy chains, which is referred to as C/A dynein (*17*).

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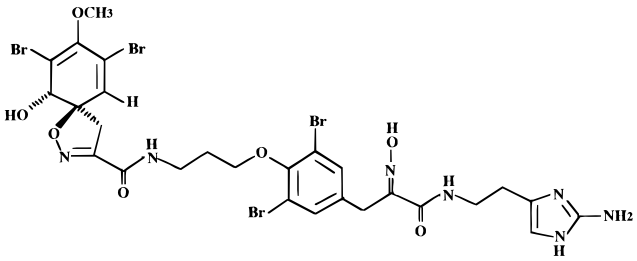

FIGURE 1: Chemical structure of purealin.

The C/A dynein possesses properties distinct from 21S dynein (*18*). On the other hand, there are several reports suggesting a functional diversity of the heavy chains within the dynein arms. *In vitro* experiments demonstrate that Aβ heavy chain but not Aα chain, dissociated from 21S dynein, can produce movement of microtubules at a velocity similar to that produced by intact dynein (*19*, *20*). The functional diversity of the heavy chains is also suggested from the studies with *Chlamydomonas* mutant lacking α or β heavy chain of outer arms (*8*, *21*). Thus, although the every heavy chain in the dynein arms would have a site for ATP hydrolysis, a single heavy chain could fully be functional as a motor. Therefore, it is assumed that only a part of the ATPase activity of the dynein arms is directly related to the flagellar movement.

Selective modulators (inhibitors and/or activators) are extremely useful probes for characterizing the function of target proteins. To date, several inhibitors of the dynein ATPase and ciliary or flagellar movement are available (*22*), but none of the selective inhibitors for the ATPase activity that directly relate to the flagellar movement have been reported. In the course of our survey of biologically active substances from natural sources, purealin has been isolated from an Okinawan sea sponge, *P. purea*, as a modulator of skeletal (*23*, *24*) and smooth (*25*) muscle myosin ATPase. Here we describe for the first time that purealin selectively inhibits the dynein ATPases that function as the motor for flagellar movement.

MATERIALS AND METHODS

Chemicals. Trypsin (type III) was purchased from Sigma Chemical Co. (St. Louis, MO). Soybean trypsin inhibitor was from Cooper Biomedical Inc., Worthington Biochemicals (Freehold, NJ). ATP was from Yamasa Shoyu Co. (Tokyo, Japan). All other chemicals were obtained from commercial sources and were of analytical grade.

Purification of Purealin. Purealin (Figure 1) was isolated from an Okinawan sea sponge, *Psammaplysilla purea*. Details of the purification have been published (*26*). Purealin was dissolved in dimethyl sulfoxide, and a final concentration of dimethyl sulfoxide used in the present experiments did not exceed 0.5%. The same concentration of dimethyl sulfoxide was added for the control experiments.

Preparation of Triton-Demembranated Sperm Models and Measurement of the Motility. Spermatozoa were obtained from the sea urchin, *Anthocidaris crassispina*, by the injection of 0.5 M KCl into the body cavity and were stored on ice as "dry" sperm, which were used within 10 h. The dry sperm were diluted with 20 vol of chilled filtrated seawater to make a stock sperm suspension, which was used within 4 h. Ten microliters of this suspension was added to 490 μL of a demembranation medium consisting of 0.04% Triton X-100, 0.15 M potassium acetate, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA),[1] 2 mM dithiothreitol (DTT), and 20 mM Tris-HCl, pH 8.0, and gently swirled at room temperature. After 1 min, 10 μL of the demembranated sperm suspension was added to 490 μL of the reactivation medium consisting of 20 μM ATP, 0.15 M potassium acetate, 2 mM $MgCl_2$, 0.2 mM EGTA, 2 mM DTT, 2% (w/v) polyethylene glycol (MW 20 000), and 20 mM Tris-HCl, pH 8.0. Purealin was added to the reactivation medium prior to the addition of the demembranated sperm suspension. Forty microliters of the reactivation medium containing the demembranated sperm was mounted on a glass slide without a cover slip, and the sperm motility was examined after 5 min at 25 °C under a dark field microscope (Nikon K.K., Tokyo, Japan). The focusing was done not to close to the grass surface. The percent motility of the demembranated sperm was defined as the percentage of swimming sperm in the total number of the sperm.

Preparation of Axonemes and 21S Dynein. Axonemes were isolated from the sperm tails of the sea urchin, *A. crassispina*, without detergent treatment as described previously (*27*). Outer-arm-depleted axonemes were prepared by salt extraction of the axonemes as described previously (*17*) with slight modifications. In brief, axonemes were suspended in MET solution (4 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 μg/mL leupeptin, 0.2 mM phenylmethanesulfonyl fluoride, and 10 mM Tris-HCl, pH 8.0) containing 0.6 M NaCl, and extracted for 1 h on ice with occasional agitation. The suspension was then centrifuged at 12000*g* for 10 min. This extraction was repeated once more. The pellet fraction was used as the outer-arm-depleted axonemes. For further extraction, these axonemes were resuspended in MET solution supplemented with 0.7 M NaCl and 0.1% Triton X-100 and stood on ice for 1 h with occasional agitations. This procedure removes a part of the inner arms. The axonemes obtained after several rinses with MET solution were referred to as ST-axonemes (*27*).

21S dynein was purified from the 0.6 M NaCl extract of the axonemes. The 0.6 M NaCl extract was centrifuged at 100000*g* for 30 min and concentrated by ultrafiltration (cutoff 10 000). The extract was dialyzed against MET solution containing 0.1 M NaCl and then centrifuged on a sucrose linear density gradient (5−20% (w/v)) at 94000*g* for 23 h (*17*). After fractionation, the peak fraction of ATPase activity was used as purified 21S dynein. Protein concentration of the preparations was determined by the method of Lowry et al. (*28*), using bovine serum albumin as a standard.

Measurement of the Sliding Movement of Axonemes. Axonemes (0.3 mg/mL) were suspended in an assay solution consisting of 0.15 M potassium acetate, 4 mM $MgCl_2$, 0.5 mM EGTA, and 20 mM Tris-HCl, pH 8.0. Trypsin was added to the suspension at a final concentration of 5 μg/mL, and the suspension was kept standing for 4 min on ice. The digestion was terminated by the addition of soybean trypsin inhibitor (70 μg/mL). The sliding disintegration of the axonemes was started by the addition of 20 μM ATP at 25 °C and monitored by the turbidity changes of the

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; UV, ultraviolet; IC_{50}, 50% inhibitory concentration.

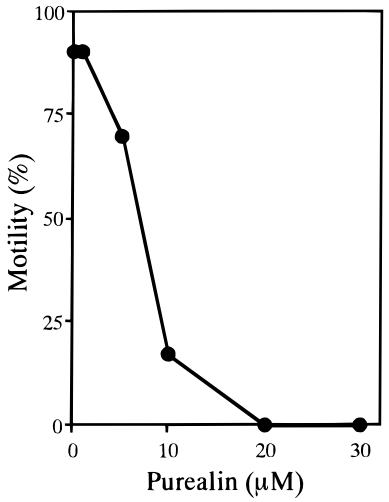

FIGURE 2: Inhibition by purealin of the motility of Triton-demembranated sperm models. The motility of the demembranated sperm was measured at 25 °C in a reactivation medium consisting of 20 μM ATP, 0.15 M potassium acetate, 2 mM $MgCl_2$, 0.2 mM EGTA, 2 mM DTT, 2% (w/v) polyethylene glycol (MW 20 000), and 20 mM Tris-HCl, pH 8.0, in the absence or presence of increasing concentrations of purealin. The percent motility of the sperm was defined as the percentage of swimming sperm in the total number of the sperm after 5 min of incubation in the reactivation medium.

suspension at 350 nm (*29*) using a Shimadzu UV-240 spectrophotometer (Shimadzu Seisakusho Co., Ltd., Kyoto, Japan). Purealin was added to the suspension immediately before the addition of ATP.

Measurement of ATPase Activity. The ATPase activity of axonemes, outer-arm-depleted axonemes, or 21S dynein was measured at 25 °C in the assay solution. The sample was preincubated in the absence of purealin and ATP for 5 min, followed by the addition of purealin and further preincubation for 5 min. The reaction was started by the addition of 1 mM ATP and stopped by adding one-third volume of cold 15% trichloroacetic acid. The amount of inorganic phosphate liberated at 0, 4, and 8 min after the addition of ATP was determined by the method of Martin and Doty (*30*).

Effect of purealin on 21S dynein ATPase activity in the presence of ST-axonemes was also examined. 21S dynein was mixed with the ST-axonemes and kept standing on ice for 10 min in a solution of 4 mM $MgCl_2$, 0.5 mM EGTA, and 20 mM Tris-HCl, pH 8.0. As controls, the ST-axonemes and the 21S dynein were separately treated in the same manner. Each sample was assayed for ATPase activity at 25 °C in the solution containing 1 mM ATP with or without purealin as described above. The amount of inorganic phosphate liberated was determined as above.

To examine the reversibility of the inhibitory action of purealin, axonemes (0.2 mg/mL) were preincubated in the assay solution with 30 μM purealin for 5 min at 25 °C and then diluted 100-fold with the solution in the presence or absence of 30 μM purealin. After the additional 5 min of incubation, ATP was added to a final concentration of 0.5 mM. The amount of inorganic phosphate liberated was determined by the method of Anner and Moosmayer (*31*). In control experiments, axonemes were preincubated without purealin and then diluted as described above.

Vanadate-Mediated Photocleavage of Axonemal Dyneins. Axonemes (0.1 mg/mL) were preincubated with or without

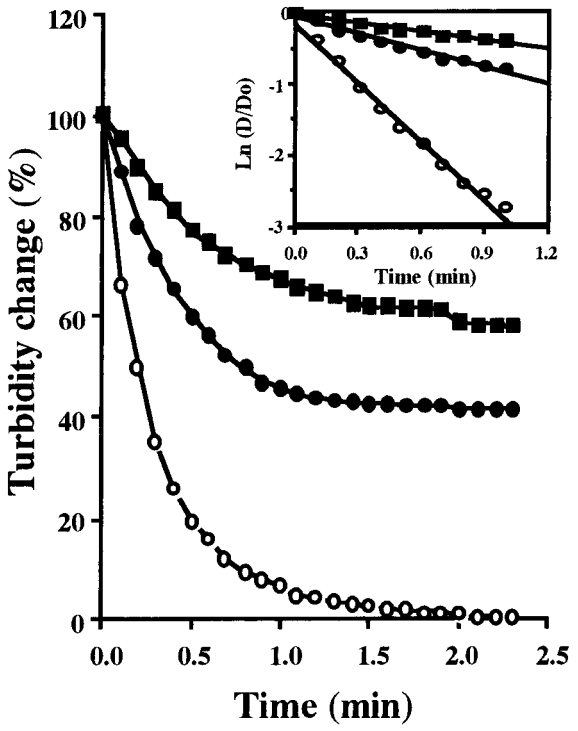

FIGURE 3: Inhibition by purealin of the sliding disintegration of axonemes. Axonemes were digested by trypsin (5 μg/mL) for 4 min on ice in an "assay" solution consisting of 0.15 M potassium acetate, 4 mM $MgCl_2$, 0.5 mM EGTA, and 20 mM Tris-HCl, pH 8.0. The digestion was terminated by the addition of soybean trypsin inhibitor (70 μg/mL). The sliding disintegration of the trypsinized axonemes was started by the addition of 20 μM ATP at 25 °C and monitored by the turbidity changes of the suspension at 350 nm in the presence of 0 or 3 (open circles), 10 (filled circles), and 30 (filled squares) μM purealin, which was added to the suspension immediately before the addition of ATP. The inset shows the rate of the turbidity changes within the first 1 min, which are −2.73, −0.78, and −0.41/min for 0 or 3, 10, and 30 μM purealin, respectively. The turbidity at the indicated time (D) is expressed relative to the initial turbidity measured before initiating sliding (D_o).

30 μM purealin for 5 min at 25 °C in the assay solution and then irradiated with ultraviolet (UV) light at 365 nm using a Manaslu UV light (Manaslu Co., Tokyo, Japan) in the presence of 0.2 mM ATP and 0.1 mM vanadate (Na_3VO_4) for 1 h on ice. The samples were electrophoresed on a 5% polyacrylamide gel in the buffer system of Laemmli (*32*) and stained with Coomassie Brilliant Blue.

RESULTS

Inhibition by Purealin of the Motility of Triton-Demembranated Sperm Models. Triton X-100-treated sperm preserved the motile apparatus devoid of plasma membrane and could swim in the presence of ATP. The motility of the demembranated sperm was examined at 5 min after the addition of the sperm into the reactivation medium including ATP with or without purealin. As shown in Figure 2, purealin inhibited the motility of the demembranated sperm in a concentration-dependent manner with an IC_{50} of 7 μM. Above 20 μM, purealin completely blocked the motility of the sperm.

Inhibition by Purealin of the Sliding Disintegration of Axonemes. In the trypsin-treated flagellar axonemes, ATP induces a sliding disintegration of the outer doublet microtubules (*33*), resulting in a decrease in the turbidity of the

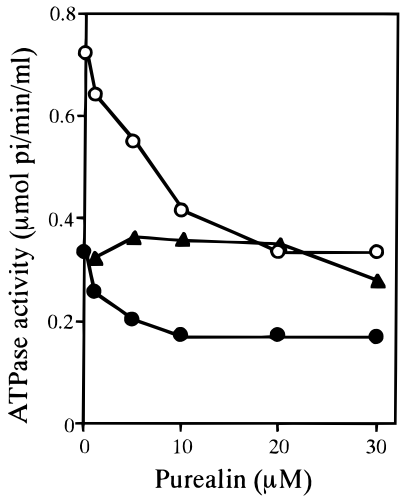

FIGURE 4: Inhibition by purealin of ATPase activity of axonemes and outer-arm-depleted axonemes. Outer-arm-depleted axonemes were prepared by 0.6 M NaCl extraction of the axonemes as described in Materials and Methods. The ATPase activity of intact (open circles) and outer-arm-depleted (filled circles) axonemes was measured at 25 °C in the assay solution containing 1 mM ATP in the absence or presence of increasing concentrations of purealin. Effect of purealin on the ATPase activity of the NaCl extract (filled triangles) was also measured as above and presented in this figure.

suspension with time (*34*). As shown in Figure 3, after the addition of ATP (20 μM) to the trypsinized axonemes of sea urchin sperm, the turbidity of the suspension decreased immediately. The maximum decrease in the turbidity was attained within 2 min. Purealin inhibited the rate (Figure 3, inset), and the magnitude of the turbidity decreases in a concentration-dependent manner. The IC_{50} for the maximum turbidity decrease was 17 μM. The rate of the turbidity decrease was reduced to one-third by 10 μM purealin.

Inhibition by Purealin of the ATPase Activity of Axonemes and Outer-Arm-Depleted Axonemes. The ATPase activity of sea urchin flagellar axonemes was around 0.7 μmol of Pi min^{-1} mg^{-1} at 1 mM ATP at 25 °C. By the extraction of outer arms with 0.6 M NaCl solution, about half of the axonemal ATPase activity was solubilized into the NaCl solution, and the another half remained in the resulting outer-arm-depleted axonemes.

Figure 4 shows the concentration dependence of the effect of purealin on these ATPase activities. The ATPase activity of intact axonemes and outer-arm-depleted axonemes decreased to 48 and 51%, respectively, of their control value on increasing the concentration of purealin up to 20 μM. Further increasing of the purealin concentration up to 30 μM did not reduce the ATPase activities. Similar results were observed even at 60 μM purealin (not shown), indicating that the inhibitory action of purealin reached a plateau above 20 μM. In contrast to the axonemal ATPases, the ATPase activity in the NaCl extract, which contained outer-arm (21S) dynein, increased by purealin to 108% of the control over a concentration range of 5−20 μM.

Figure 5 shows the effect of dilution of purealin on the inhibitory action to the specific ATPase activity of axonemes. In control experiments (Figure 5, open bars), when axonemes were 100-fold diluted with a assay solution containing 30 μM purealin, the ATPase activity revealed 57% of the control value that was measured in the absence of purealin. Next, the axonemes were preincubated with 30 μM purealin for 5 min and then diluted as above (Figure 5, filled bars) either

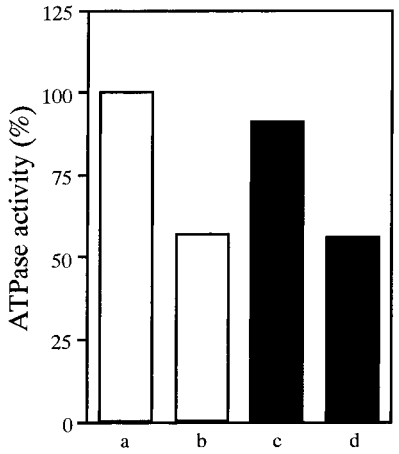

FIGURE 5: Reversible inhibitory action of purealin on axonemal ATPases. Axonemes were preincubated without (a, b) or with (c, d) 30 μM purealin for 5 min at 25 °C in the assay solution and then diluted 100-fold with the solution without (a, c) or with (b, d) 30 μM purealin. The ATPase activity was determined by the method of Anner and Moosmayer (*31*) as described in Materials and Methods and is shown as percent specific activity.

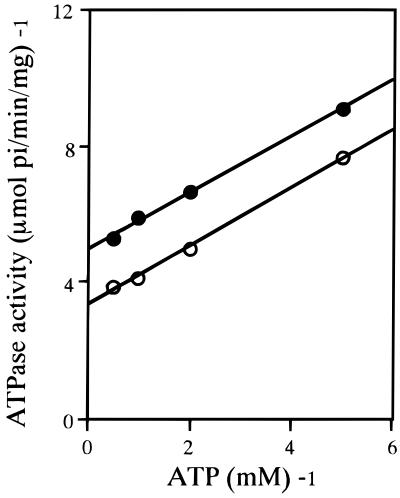

FIGURE 6: Effect of purealin on the double-reciprocal plot of axonemal ATPases. The ATPase activity of axonemes as a function of ATP concentrations was measured in the absence (open circles) or presence (filled circles) of 30 μM purealin under the same conditions as those in Figure 4.

in the presence or absence of 30 μM purealin. In the presence of purealin, the ATPase activity of the preincubated axonemes revealed 56% of the control value. However, when diluted in the absence of purealin to a final purealin concentration of 0.3 μM, the activity returned to 91% of the control value, indicating that the inhibitory action of purealin is reversible.

The effect of purealin on the ATPase activity of axonemes as a function of ATP concentration was also examined. As shown in Figure 6, the double-reciprocal plots showed a reduced V_{max} with no significant change in slope by the presence of purealin, suggesting that purealin does not compete with ATP at the catalytic site of the enzyme.

In these ATPase assays, we determined inorganic phosphate liberated at 0, 4, and 8 min after the addition of ATP. The amount of inorganic phosphate liberated was a liner function with time at any concentrations of purealin used (not shown), suggesting that purealin inhibits axonemal ATPase activity within 5 min.

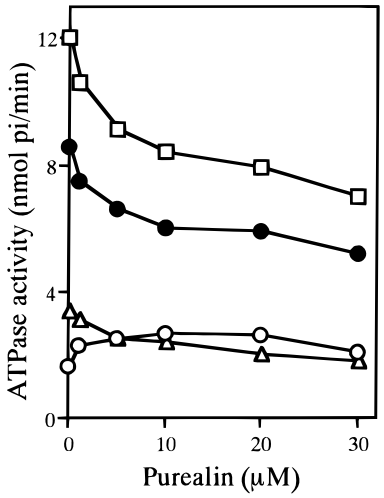

FIGURE 7: Effect of purealin on the ATPase activity of 21S dynein in the presence of ST-axonemes. The ATPase activity of 21S dynein alone (open circles), ST-axonemes alone (open triangles), and the mixture of the 21S dynein and the ST-axonemes (open squares) were measured at 25 °C in a medium consisting of 4 mM $MgCl_2$, 0.5 mM EGTA, 1 mM ATP, and 20 mM Tris-HCl, pH 8.0, in the presence of various concentrations of purealin. The final concentrations of the 21S dynein and of the ST-axonemes were 12 and 180 μg/mL, respectively. The 21S dynein ATPase activity stimulated by the ST-axonemes (closed circles) was calculated by subtracting the ATPase activity of the ST-axonemes alone from that of the mixture of the 21S dynein and the ST-axonemes, as described previously (*27*).

Effect of Purealin on the ATPase Activity of 21S Dynein in the Presence of ST-Axonemes. As shown previously (*27*), the ATPase activity of 21S dynein was increased to more than 5-fold in the presence of ST-axonemes (Figure 7, closed circles). This increase was inhibited in a manner dependent on the purealin concentration: the activity in the presence of the ST-axonemes was decreased to 60% in the presence of 30 μM purealin. The ATPase activity of the ST-axonemes alone was also reduced by purealin (Figure 7, open triangles). On the other hand, the ATPase activity of the 21S dynein alone was slightly increased by purealin (Figure 7, open circles), indicating that the stimulation of the ATPase activity in the NaCl extract by purealin as shown in Figure 4 is due to the activation of 21S dynein ATPase by purealin.

Effect of Purealin on the Vanadate-Mediated Photocleavage of Axonemal Dyneins. SDS−polyacrylamide gel electrophoresis of axonemes showed four polypeptide bands of dynein heavy chains, referred to as C, A, D, and B in order of increasing rate of migration (Figure 8). These heavy chains were cleaved when the axonemes were irradiated by UV light only in the presence of both ATP and vanadate (Figure 8A) and generated the heavy fragments (HUV) and light fragments (LUV) as shown previously by Gibbons and Gibbons (*14*). Purealin (30 μM) did not affect the photocleavage of the heavy chains of axonemal dyneins (Figure 8B).

DISCUSSION

It is well known that dynein ATPases are inhibited by unhydrolyzable ATP analogs such as β,γ-methyleneadenosine 5′-triphosphate (*35*), erythro-9-3-2(hydroxynonyl) adenine (*36*), and vanadate (*37*). The inhibitory effects of purealin on the dynein ATPases are quite different from those of these inhibitors.

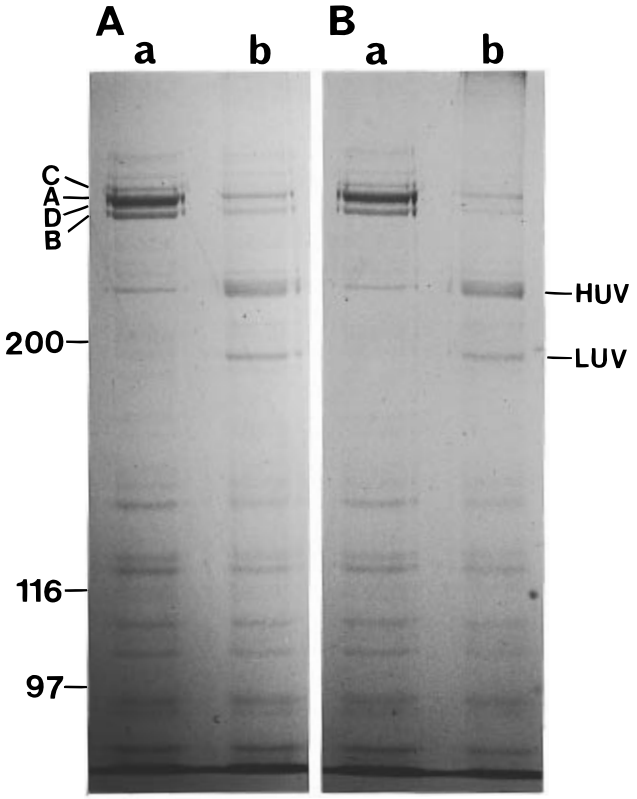

FIGURE 8: Effect of purealin on the vanadate-mediated photocleavage of axonemal dyneins. Axonemes were preincubated with (B) or without (A) 30 μM purealin for 5 min at 25 °C in the assay solution and further incubated in the presence of 0.2 mM ATP and 0.1 mM vanadate (Na_3VO_4) for 1 h on ice with (b) or without (a) irradiation of UV light at 365 nm. The samples were electrophoresed on a 5% polyacrylamide gel in the buffer system of Laemmli (*32*) and stained with Coomassie Brilliant Blue. In this study, the nomenclature by Gibbons and Gibbons (*14*) is used for dynein heavy chains and photocleaved fragments; C, A, D, and B, dynein heavy chains; HUV, heavy fragments; LUV, light fragments.

Purealin blocked the flagellar motility of Triton-demembranated sea urchin sperm within 5 min at the concentration above 20 μM (Figure 2). In the similar concentration range, purealin slowed the velocity of the ATP-induced decrease in the turbidity of trypsinized axonemes for first 1 min (Figure 3). The maximum decrease in the turbidity, which was attained within 2 min, was also inhibited by purealin. Kamimura et al. (*29*) has shown that the decrease in the turbidity is strongly related to the sliding disintegration of the outer doublet microtubules. These observations suggest that purealin slows the sliding velocity of the axonemes and then completely blocks the sliding movement within a few minutes.

Essential to the sliding movement are the two types of dynein ATPase, which constitute the two rows of inner and outer arms on the A-subfibers of the doublet microtubules and produce force by interacting with adjacent B-subfibers in the presence of Mg-ATP (*1*, *2*). The outer arms can be extracted with high salt solution as a 21S dynein (*15*). Since the extraction almost completely removes the outer row of arms but leaves the inner row of arms intact (*12*, *15*), the remaining ATPase activity of the outer-arm-depleted axonemes is mainly attributed to the inner dynein arms. In this experiment, ATPase activity of outer-arm-depleted axonemes was about half that of intact axonemes, indicating a 1:1 contribution of inner and outer dynein arms to the ATPase activity of the intact axonemes. Purealin inhibited

maximally 50% of the ATPase activity of the outer-arm-depleted axonemes (Figure 4), indicating that half the activity of inner arms is sensitive to purealin. The extent of the inhibition corresponded to about 25% of the ATPase activity of intact axonemes. Therefore, if purealin does not inhibit the ATPase activity of outer-arm dynein in the intact axonemes, the axonemal ATPase activity may be reduced by purealin to approximately 75% at maximum. However, purealin inhibited the ATPase activity of the intact axonemes by 50% at maximum (Figure 4). These results suggest that purealin inhibits half the ATPase activity of inner and outer dynein arms, respectively.

Inhibition by purealin of the ATPase activity of axonemes was reversed by dilution (Figure 5), indicating that the inhibition was due to the reversible binding of purealin to the axonemal dyneins. We have previously reported that an inhibitory action of purealin is observed only on the enzymes related to adenine nucleotides, but not on several phosphatases and lactate dehydrogenase (*24*). Therefore, purealin is suggested to have some interaction with those enzymes at the nucleotide binding sites. In the dynein ATPases, putatively four nucleotide binding sites (P-loops) in the heavy chains have been predicted earlier on the basis of their amino acid sequences (*13*). The P-loop corresponding to the probable catalytic ATP-binding site (P1) can be identified by its location close to or at the V1 site of vanadate-mediated photocleavage (*38*), but the functional aspects of other additional noncatalytic sites are not yet known. In the present experiments, the effect of purealin on the double-reciprocal plot of axonemal ATPases showed that the inhibition was not a competitive type. Furthermore, purealin did not affect the vanadate-mediated photocleavage of the axonemal dyneins. These results suggest that purealin binds to a site other than a catalytic ATP-binding site in the axonemal dyneins and modulates the ATP hydrolysis at the catalytic site.

On the other hand, such inhibitory action of purealin was not observed on the ATPase activity of 0.6 M NaCl extract from the axonemes (Figure 4) and of isolated outer (21S) arm dyneins (Figure 7). In contrast to the axonemal dynein ATPases, purealin did not inhibit but rather activated these solubilized dynein ATPases. Such activation seems to conflict with the inhibitory action of purealin on the flagellar motility or the sling movement of axonemes and is, therefore, not a physiologically significant action of purealin. It has been revealed that the 21S dynein rebinds to outer doublets in the axonemes and the ATPase activity is stimulated (*15*, *27*). The stimulated ATPase activity of the 21S dynein by the axonemes was inhibited by purealin in a manner similar to that of the intact axonemes or the outer-arm-depleted axonemes. These results suggest that the action of purealin on the axonemal dynein ATPases, but not on the solubilized dynein ATPases, is correlated with the function of dynein arms in the axonemes.

In our biochemical experiments reported here, the ATPase activity of axonemal dyneins can be divided into two classes on the basis of the sensitivity to purealin. Furthermore, a quite important observation is that purealin can block the sliding movement of flagellar axonemes. A probable explanation of these results has been that the purealin-sensitive ATPase activity of the dynein arms plays an essential role in generating the sliding movement of flagellar axonemes. There are several reports consistent with our results. Axonemal dyneins form stable cross-bridges, called "rigor" state, between adjacent outer doublet microtubules in the absence of ATP. This rigor state can be broken by the addition of ATP. In sea urchin sperm flagella, outer-arm dynein consists of two distinct heavy chains called Aα and Aβ, both of which have ATPase activity (*15*, *16*). *In vitro* experiments have demonstrated (*19*, *20*) that the Aα chain produces the rigor bond but does not function as a motor. In contrast, the Aβ chain is fully functional as the motor, producing *in vitro* microtubule gliding velocities almost equivalent to those produced by intact dynein, but does not bind microtubules in the absence of ATP. These observations suggest a functional diversity of the ATPase activity of outer dynein arms on the sliding movement of the axonemes. On the other hand, in the inner-arm dyneins, the heavy chain compositions and their functional diversity are not fully understood. Here, we demonstrated that purealin can inhibit half the ATPase activity of inner-arm dyneins in the axonemes, suggesting that the functional diversity also occurs within the inner dynein arms. However, further studies are needed to clarify the ATPases, whose activity is inhibited by purealin in the axonemes.

In conclusion, our results suggest that purealin selectively inhibits the dynein ATPase functioning as the motor in axonemes. Thus, purealin may provide a useful chemical tool for studying the function of dynein arms.

REFERENCES

1. Gibbons, I. R. (1981) *J. Cell Biol. 91*, 107s−123s.
2. Johnson, K. A. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 161−188.
3. Gibbons, I. R. (1988) *J. Biol. Chem. 263*, 15837−15840.
4. Mitchell, D. R. (1994) *Int. Rev. Cytol. 155*, 141−180.
5. Goodenough, U. W., and Heuser, J. E. (1989) in *Cell Movement*, Vol. 1 (Warner, F. D., Satir, P., and Gibbons, I. R., Eds.), pp 121−140, Alan R. Liss, Inc., New York.
6. Goodenough, U. W., and Heuser, J. E. (1985) *J. Cell Biol. 100*, 2008−2018.
7. Piperno, G., Ramanis, Z., Smith, E. F., and Sale, W. S. (1990) *J. Cell Biol. 110*, 379−389.
8. Brokaw, C. J., and Kamiya, R. (1987) *Cell Motil. Cytoskel. 8*, 68−75.
9. Kamiya, R., Kurimoto, E., Sakakibara, H., and Okagaki, T. (1989) in *Cell Movement*, Vol. 1 (Warner, F. D., Satir, P., and Gibbons, I. R., Eds.), pp 209−218, Alan R. Liss, Inc., New York.
10. Kamiya, R., Kurimoto, E., and Muto, E. (1991) *J. Cell Biol. 112*, 441−447.
11. Gibbons, B. H., and Gibbons, I. R. (1973) *J. Cell Sci. 13*, 337−357.
12. Fox, L. A., and Sale, W. S. (1987) *J. Cell Biol. 105*, 1781−1787.
13. Asai, D. J., and Lee, S.-W. (1995) *Mol. Cells 5*, 299−305.
14. Gibbons, B. H., and Gibbons, I. R. (1987) *J. Biol. Chem. 262*, 8354−8359.
15. Gibbons, I. R., and Fronk, E. (1979) *J. Biol. Chem. 254*, 187−196.
16. Tang, W. Y., Bell, C. K., Sale, W. S., and Gibbons, I. R. (1982) *J. Biol. Chem. 257*, 508−515.
17. Yokota, E., and Mabuchi, I. (1994) *J. Cell Sci. 107*, 345−351.
18. Yokota, E., and Mabuchi, I. (1994) *J. Cell Sci. 107*, 353−361.
19. Sale, W. S., and Fox, L. A. (1988) *J. Cell Biol. 107*, 1793−1797.
20. Moss, A. G., Gatti, J.-L., and Witman, G. B. (1992) *J. Cell Biol. 118*, 1177−1188.
21. Sakakibara, H., Mitchell, D. R., and Kamiya, R. (1991) *J. Cell Biol. 113*, 615−622.

22. Shimizu, T. (1995) in *Methods in Cell Biology*, Vol. 47 (Dentler, W., and Witman, G., Eds.), pp 497−501, Academic Press, Inc., New York.
23. Nakamura, Y., Nakamura, H., Wu, H., Kobayashi, J., and Ohizumi, Y. (1985) *Jpn. J. Pharmacol. 39*, 330.
24. Nakamura, Y., Kobayashi, M., Nakamura, H., Wu, H., Kobayashi, J., and Ohizumi, Y. (1987) *Eur. J. Biochem. 167*, 1−6.
25. Takito, J., Nakamura, H., Kobayashi, J., and Ohizumi, Y. (1986) *J. Biol. Chem. 261*, 13861−13865.
26. Nakamura, H., Wu, H., Kobayashi, J., Nakamura, Y., Ohizumi, Y., and Hirata, Y. (1985) *Tetrahedron Lett. 26*, 4517−4520.
27. Yokota, E., Mabuchi, I., and Sato, H. (1987) *J. Biochem. 102*, 31−41.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
29. Kamimura, S., Nakanishi, M., Yano, M., and Shimizu, H. (1986) *Exp. Cell Res. 163*, 186−190.
30. Martin, M., and Doty, M. (1949) *Anal. Chem. 21*, 965−967.
31. Anner, B., and Moosmayer, M. (1975) *Anal. Biochem. 65*, 305−309.
32. Laemmli, U. K. (1970) *Nature 227*, 680−685.
33. Summers, K. E., and Gibbons, I. R. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3092−3096.
34. Summers, K. E., and Gibbons, I. R. (1978) *J. Cell Biol. 58*, 618−629.
35. Penningroth, S. M., and Witman, G. B. (1978) *J. Cell Biol. 79*, 827−832.
36. Penningroth, S. M., Cheung, A., Bouchard, P., Gagnon, C., and Bardin, W. (1982) *Biochem. Biophys. Res. Commun. 104*, 234−240.
37. Gibbons, I. R., Cosson, M. P., Evans, J. A., Gibbons, B. H., Houck, B., Martinson, K. H., Sale, W. S., and Tang, W. Y. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2220−2224.
38. Gibbons, I. R., and Mocz, G. (1991) *Methods Enzymol. 196*, 428−442.

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