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Inhibitory Regulation of Higher-Plant Myosin by Ca^{2+} Ions[1]

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Myosin isolated from the pollen tubes of lily (*Lilium longiflorum*) is composed of a 170-kD heavy chain (E. Yokota and T. Shimmen [1994] Protoplasma 177: 153–162). Both the motile activity in vitro and the F-actin-stimulated ATPase activity of this myosin were inhibited by Ca^{2+} at concentrations higher than 10^{-6} M. In the Ca^{2+} range between 10^{-6} and 10^{-5} M, inhibition of the motile activity was reversible. In contrast, inhibition by more than 10^{-5} M Ca^{2+} was not reversible upon Ca^{2+} removal. An 18-kD polypeptide that showed the same mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as that of spinach calmodulin (CaM) was present in this myosin fraction. This polypeptide showed a mobility shift in sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a Ca^{2+}-dependent manner. Furthermore, this polypeptide was recognized by antiserum against spinach CaM. By immunoprecipitation using antiserum against the 170-kD heavy chain, the 18-kD polypeptide was coprecipitated with the 170-kD heavy chain, provided that the Ca^{2+} concentration was low, indicating that this 18-kD polypeptide is bound to the 170-kD myosin heavy chain. However, the 18-kD polypeptide was dissociated from the 170-kD heavy chain at high Ca^{2+} concentrations, which irreversibly inhibited the motile activity of this myosin. From these results, it is suggested that the 18-kD polypeptide, which is likely to be CaM, is associated with the 170-kD heavy chain as a light chain. It is also suggested that this polypeptide is involved in the regulation of this myosin by Ca^{2+}. This is the first biochemical basis, to our knowledge, for Ca^{2+} regulation of cytoplasmic streaming in higher plants.

In plant cells cytoplasmic streaming plays an essential role in intracellular transport of organelles and molecules. The motive force for cytoplasmic streaming is generated by the active sliding of myosin, which is associated with organelles, along actin filaments (Williamson, 1976; Staiger and Schliwa, 1987; Shimmen and Yokota, 1994). Physiological studies of Ca^{2+} regulation of cytoplasmic streaming have been carried out extensively in the algae family Characeae. In characean cells the cytoplasmic concentration of Ca^{2+} increases to more than 10^{-6} M when an action potential is generated at the plasma membrane (Williamson and Ashley, 1982). Concomitantly with this Ca^{2+} increase, cytoplasmic streaming stops transiently (Hayama et al., 1979; Williamson and Ashley, 1982). Microinjection of Ca^{2+} into the cytoplasm of an internodal cell of *Nitella axillitormis* causes a reversible inhibition of cytoplasmic streaming (Kikuyama and Tazawa, 1982). The membrane-permeabilized cell model prepared from characean cells provides direct evidence that cytoplasmic streaming is reversibly inhibited by an elevation of Ca^{2+} to 10^{-6} M (Tominaga et al., 1983). For the molecular mechanism, involvement of myosin phosphorylation has been suggested (Tominaga et al., 1987; McCurdy and Harmon, 1992a, 1992b): Myosin is phosphorylated by a Ca^{2+}-dependent protein kinase, which is inactivated by high Ca^{2+}.

The inhibitory effect of Ca^{2+} on cytoplasmic streaming has also been reported in some higher-plant cells such as lily (*Lilium longiflorum*) pollen tubes (Kohno and Shimmen, 1988b), stamen hair cells of *Tradescantia* (Doree and Picard, 1980), trichome cells of tomato (Woods et al., 1984), and leaf cells of *Vallisneria gigantia* (Takagi and Nagai, 1986). It has been suggested that pollen tube myosins have an inhibitory Ca^{2+} sensitivity. This is based on the facts that, along characean actin cables, movement of pollen tube organelles is inhibited by Ca^{2+} (Kohno and Shimmen, 1988a) and that characean actin cables are not equipped with a Ca^{2+}-sensitizing mechanism (Shimmen and Yano, 1986). Biochemical studies of the molecular mechanism for Ca^{2+} regulation of the myosin activity responsible for cytoplasmic streaming are scarce in higher-plant cells because of difficulties in isolating myosin. Although Ca^{2+}-sensitive myosin was isolated from tomato fruits, its F-actin-activated ATPase activity is stimulated by Ca^{2+} (Vahey et al., 1982). Thus, it is unlikely that this myosin is involved in cytoplasmic streaming.

Recently, we isolated a higher-plant myosin composed of a 170-kD heavy chain from the pollen tubes of lily (Yokota and Shimmen, 1994). In the present study this myosin is referred to as the 170-kD myosin. This 170-kD myosin was able to translocate F-actin in vitro at a velocity similar to that of cytoplasmic streaming in living pollen tubes of lily, and its ATPase was stimulated by F-actin (Yokota and Shimmen, 1994). Taken together, these biochemical results and immunocytochemical studies using antibodies against the heavy chain of 170-kD myosin (Yokota et al., 1995a, 1995b) suggest that this myosin is generally distributed in higher plants and that it is involved in generating the motive force for cytoplasmic streaming. In the present study we have shown that the activities of 170-kD myosin are inhibited by Ca^{2+} and suggest that CaM, a light chain of this myosin, is involved in Ca^{2+} regulation. To our

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Abbreviations: CaM, calmodulin; RP, rhodamine-phalloidin.

knowledge, this is the first description of the biochemical basis for the Ca^{2+} regulation of cytoplasmic streaming in higher plants.

MATERIALS AND METHODS

Isolation of 170-kD Myosin and CaM from Lily Pollen and of Actin from Skeletal Muscle

The isolation of 170-kD myosin from germinated pollen of lily (*Lilium longiflorum)* was carried out according to the method described previously (Yokota and Shimmen, 1994). The 170-kD myosin was isolated from a crude extract of pollen tubes by cosedimentation with F-actin and purified successively by a hydroxylapatite column (Pharmacia LKB) and a Sephacryl S-300 gel-filtration column (Pharmacia LKB). Finally, the 170-kD myosin was suspended in a solution containing 0.1 M KCl, 1 mM EGTA, 4 mM $MgCl_2$, 50 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 30 mM Pipes-KOH (pH 7.0).

CaM was extracted from the TCA precipitate of germinating lily pollen according to the method of Yazawa et al. (1980) and further purified by a fluphenazine-affinity column (Kakiuchi et al., 1981). Unless otherwise noted, each procedure was carried out at 0°C to 4°C. In each column step described below, the fractions, including CaM, were monitored by SDS-PAGE. The germinating pollen grains were suspended in 4% (w/v) TCA and then homogenized by 15 strokes with a motor-driven glass-Teflon homogenizer. After the sample was centrifuged at 9,000*g* for 10 min, the pellet was suspended in a solution containing 5 mM EGTA, 100 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 20 mM Tris-HCl (pH 7.5). The pH of the suspension was adjusted to 7.5 with NaOH. After incubation on ice for 30 min, the suspension was centrifuged at 25,000*g* for 30 min. The supernatant was applied to an ion-exchange column (model DE-52, Whatman) preequilibrated with ET solution (0.2 mM EGTA, 50 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 20 mM Tris-HCl [pH 7.5]) supplemented with 0.14 M ammonium sulfate. After the column was washed with the preequilibrated solution, the adsorbed materials were eluted with the ET solution supplemented with 0.3 M ammonium sulfate.

Fractions containing CaM were pooled and diluted 1.5-fold with ET solution. After the addition of $CaCl_2$ (final concentration at 0.4 mM), the dilutant was applied to a column of Sepharose 6B (Pharmacia) conjugated with fluphenazine (Sigma) according to the method of Kakiuchi et al. (1981). The column was first washed with CT solution (0.2 mM $CaCl_2$, 50 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 20 mM Tris-HCl [pH 7.5]) and subsequently with the CT solution supplemented with 0.5 M NaCl. CaM was then eluted with a solution containing 0.5 M NaCl, 2 mM EGTA, 50 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 20 mM Tris-HCl (pH 7.5). After dialysis against a solution containing 60 mM KCl, 50 μg/mL leupeptin, 0.5 mM PMSF, 1 mM DTT, and 30 mM Pipes-KOH (pH 7.0), CaM was stored at −80°C until use.

F-actin used for the cosedimentation procedure, the in vitro motility assay, and the ATPase activity of 170-kD myosin was prepared from chicken breast muscle according to the method of Kohama (1981).

Motility Assay in Vitro

KEMP solution (30 mM KCl, 5 mM EGTA, 6 mM $MgCl_2$ and 30 mM Pipes-KOH [pH 7.0]) was used for the motility assay in vitro. $CaCl_2$ was added to the KEMP solution to modify $[Ca^{2+}]$, and free $[Ca^{2+}]$ was calculated from the dissociation constants by using the computer analysis program (Kohno and Shimmen, 1988a). The pH of the KEMP solution containing various $[Ca^{2+}]$ was finally adjusted to 7.0 with KOH.

A coverslip was treated with 0.2% (w/v) collodion dissolved in isopentyl acetate and then air dried. Sixty microliters of 170-kD myosin (45–65 μg/mL) was applied to a piece of Parafilm (American National Can, Neenah, WI), and the collodion-coated coverslip was laid on the drop of myosin. After 5 min on ice, the surface of the coverslip was rinsed with a washing solution prepared by adding 1 mM ATP, 2 mM DTT, and various concentrations of $CaCl_2$ to the KEMP solution. To make a flow chamber with a volume of approximately 10 μL, a small amount of petroleum jelly was applied to the two opposite edges of the coverslip. The coverslip was then placed on a glass slide. The flow chamber was perfused two times with 60 μL of the washing solution and subsequently with 60 μL of an assay medium (1 mM ATP, 0.3 μg/mL RP-labeled F-actin, 0.216 mg/mL glucose oxidase, 36 μg/mL catalase, 4.5 mg/mL Glc, 0.6% methyl cellulose, 100 mM DTT, and various concentrations of $CaCl_2$ in the KEMP solution). RP-labeled F-actin was prepared by incubating F-actin with RP (Molecular Probes, Eugene, OR), according to the method of Kohno et al. (1991). Movement of actin filaments over the surface of the coverslip was observed under a fluorescence microscope (model BH2, Olympus) equipped with epifluorescence optics (model BH2-RFC, Olympus).

Images were recorded on videotapes with a high-sensitivity television camera (model C2400–08 SIT, Hamamatsu Photonics K.K., Sunayamacho, Hamamatsu, Japan) and a video recorder (model NV-FS65, Nihon National Instruments K.K., Tokyo, Japan). Motile activities were assessed in two ways. First, the percentage of translocated RP-labeled F-actin was determined by checking 500 to 700 RP-labeled F-actins located at the surface of the coverslip. When an RP-labeled F-actin moved continuously along its long axis for a distance longer than 5 μm, it was defined as being translocated by myosin. Second, the velocity of translocation of RP-labeled F-actin was determined. In general, velocities for at least 40 RP-labeled F-actin were measured under each condition.

Measurement of Myosin ATPase Activity

The ATPase activity of 170-kD myosin in the presence or absence of F-actin at 25°C was measured with the assay medium used for the motility assay: KEMP solution supplemented with 0. 5 mM ATP and various concentrations of $CaCl_2$. The final protein concentrations were 60 μg/mL for F-actin and 3 μg/mL for 170-kD myosin. The amount of Pi

liberated was determined according to the method of Anner and Moosmayer (1975). The ATPase activity of F-actin alone was also measured under the same conditions. To determine the F-actin-stimulated ATPase activity of myosin, the value of Pi liberation in the presence of F-actin alone was subtracted from that in the presence of both 170-kD myosin and F-actin.

Immunoprecipitation using Antiserum against the 170-kD Heavy Chain

Two microliters of rabbit antiserum raised against the 170-kD heavy chain (Yokota and Shimmen, 1994) was added to 200 μL of the myosin (50 μg/mL) fraction. As a control, preimmune serum was used instead of antiserum. The mixture was kept on ice for 1 h. Ten microliters of protein A-conjugated Sepharose beads (Pharmacia) was then added to the mixture. After further incubation for 1 h on ice, the sample was centrifuged at 500*g* for 3 min. The resultant pellet was washed three times with KEMP solution supplemented with 0.05% (v/v) Tween 20. The final pellet containing proteins bound to Sepharose beads was resuspended in SDS-PAGE sample buffer. An aliquot of the sample was subjected to SDS-PAGE on a 6% polyacrylamide gel to detect the presence of the 170-kD heavy chain. Another aliquot was subjected to immunoblot using antiserum against CaM.

The effect of Ca^{2+} on the immunoprecipitate was examined by the following two experiments. First, the myosin fraction was mixed with antiserum containing 1.5 mM $CaCl_2$, followed by the addition of Sepharose beads. Then, the beads added to the mixture were treated as described above. Second, the myosin fraction was mixed with the antiserum without 1.5 mM $CaCl_2$ and then with Sepharose beads. The beads added to the mixture were then washed with KEMP solution supplemented with 0.05% Tween 20 and various concentrations of Ca^{2+}. The proteins bound to the beads were analyzed by SDS-PAGE and immunoblotting, as described above.

Immunoblotting

After SDS-PAGE, proteins in the gel were electrophoretically transferred to a PVDF-nitrocellulose membrane (Millipore) according to the method of Towbin et al. (1979). The nitrocellulose membrane was blocked using PBS containing 2% BSA and 2% lamb serum for 1 h and then incubated with the primary antiserum against the 170-kD heavy chain (Yokota and Shimmen, 1994) or CaM (Muto and Miyachi, 1984), which was diluted 2000- or 4000-fold, respectively, with PBS supplemented with 1% BSA and 0.05% Tween 20. The detection of antibodies on the nitrocellulose membrane by anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) was carried out according to the method described previously (Yokota et al., 1995a). A spinach CaM (Sigma) was used as a positive control for the anti-CaM antiserum.

Other Methods

SDS-PAGE was performed according to the method of Laemmli (1970). Protein bands were visualized by Coomassie brilliant blue staining. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard.

RESULTS

Effect of Ca^{2+} on the Activities of 170-kD Myosin

In the presence of EGTA, RP-labeled F-actin was translocated smoothly and continuously with an average velocity of 6.2 μm/s over the glass surface coated with 170-kD

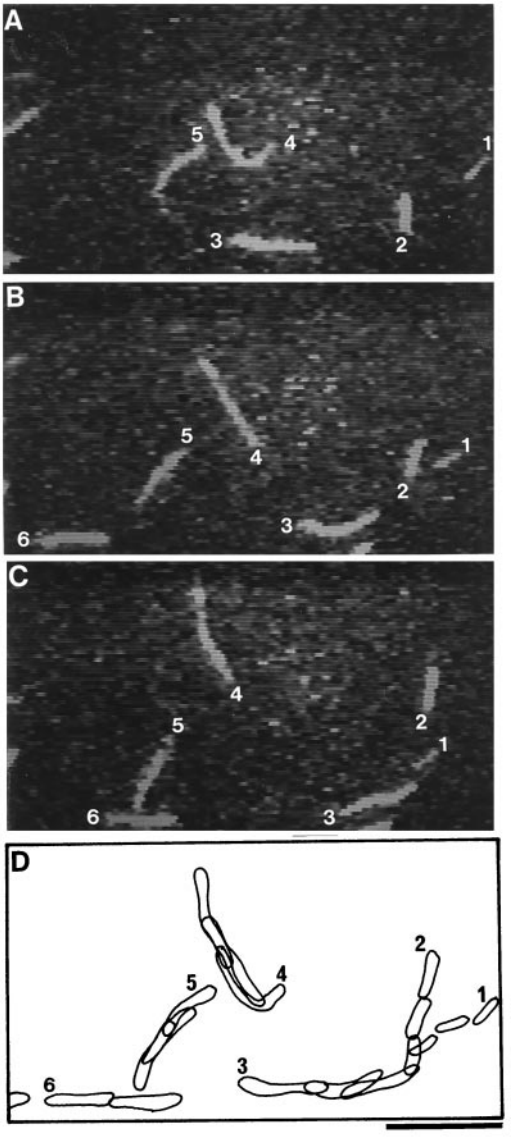

Figure 1. Sequential photographs (A–C) of moving RP-labeled F-actins over a glass surface coated with 170-kD myosin in the presence of EGTA. Photographs (A–C) were taken at time intervals of 0.66 s. Traces of the six RP-labeled F-actins (1–6) shown in A to C are superimposed in D. The bar represents 10 μm.

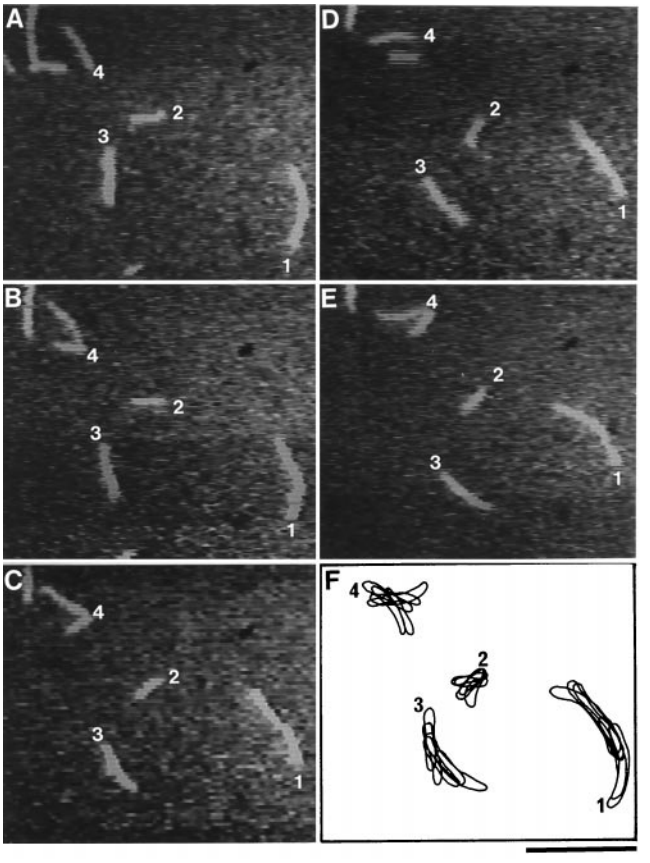

Figure 2. Sequential photographs (A–E) of moving RP-labeled F-actins over a glass surface coated with 170-kD myosin in the presence of 10^{-5} M free Ca^{2+}. Photographs (A–E) were taken at time intervals of 0.66 s. The traces of four RP-labeled F-actins (1–4) shown in A to E are superimposed in F. The bar represents 10 μm.

myosin (Fig. 1). However, the motile activity was greatly affected when the Ca^{2+} concentration was increased to more than 10^{-6} M. Some RP-labeled F-actin filaments that had been translocating stopped temporarily for a few seconds and then began translocating again (RP-labeled F-actin filaments numbered 1 and 3 in Fig. 2). Other RP-labeled F-actin showed Brownian motion (RP-labeled F-actin numbered 2 and 4 in Fig. 2). In some cases, RP-labeled F-actin detached suddenly from the glass surface (data not shown). These results suggest that the interaction of RP-labeled F-actin with 170-kD myosin is weak in the presence of Ca^{2+}. RP-labeled F-actin that moved more than 5 μm along its long axis was judged to be translocated by myosin. The percentages of translocated RP-labeled F-actin among those located at the surface of the coverslip are shown in Figure 3.

Figure 3A shows the motile activity of 170-kD myosin in vitro as a function of [Ca^{2+}]. Both the percentage of translocated RP-labeled F-actin and the velocity of translocation were reduced by [Ca^{2+}] higher than 10^{-6} M. The reversibility of the Ca^{2+} inhibition was examined (Fig. 3B). After the percentage of translocated RP-labeled F-actin was examined in the presence of Ca^{2+} at various concentrations, the flow cells were perfused with an assay medium without a supplement of Ca^{2+}, and the percentage of translocated RP-labeled F-actin was again examined. Ca^{2+} at 2.5 μM (pCa [$-\log$ [Ca^{24}]]-5.6) significantly decreased the percentage of translocated RP-labeled F-actin (Fig. 3B). The motile activity that had been inhibited by Ca^{2+} at 10^{-6} to 10^{-5} M was recovered by perfusion with an assay medium without $CaCl_2$. However, only partial recovery was observed after inhibition by 10^{-4} M Ca^{2+}.

Next, we measured the ATPase activity of 170-kD myosin in the presence or absence of F-actin as a function of [Ca^{2+}] (Fig. 4). The ATPase assay was carried out using the same buffer as that of the motility assay. The ATPase activity of 170-kD myosin alone showed similar values at all [Ca^{2+}] examined (Fig. 4, ●). It was stimulated by F-actin up to 20- to 30-fold at low Ca^{2+}. However, it was only partially activated at [Ca^{2+}] higher than 10^{-6} M, i.e. F-actin-stimulated myosin ATPase was inhibited at higher [Ca^{2+}].

Identification of CaM in Pollen Tube Myosin

Results in Figure 5 suggest that the myosin fraction contains CaM. In the myosin fraction two prominent bands, 34 kD (Fig. 5A, arrowhead 1) and 18 kD (Fig. 5A,

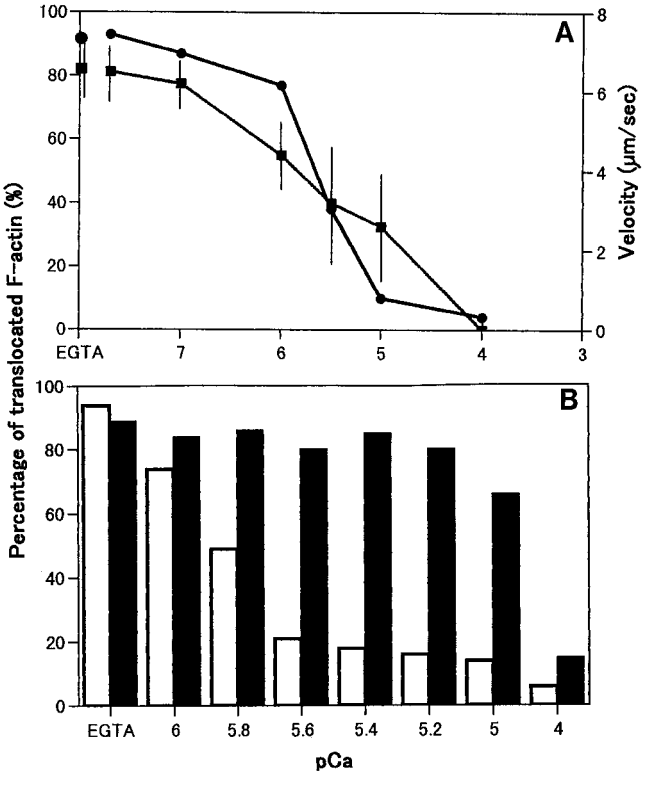

Figure 3. The effect of Ca^{2+} on the motile activity of 170-kD myosin. A, Effect of Ca^{2+} on the percentage of translocated RP-labeled F-actin (●) and velocity of translocation (■). B, Reversibility of the Ca^{2+} inhibition of 170-kD myosin. The percentage of translocated RP-labeled F-actin was measured using an assay medium containing Ca^{2+} of various concentrations (white bars). After the flow chamber was perfused with an assay medium lacking $CaCl_2$, the percentage of translocated RP-labeled F-actin was again measured (black bars). The results presented are typical of three separate experiments.

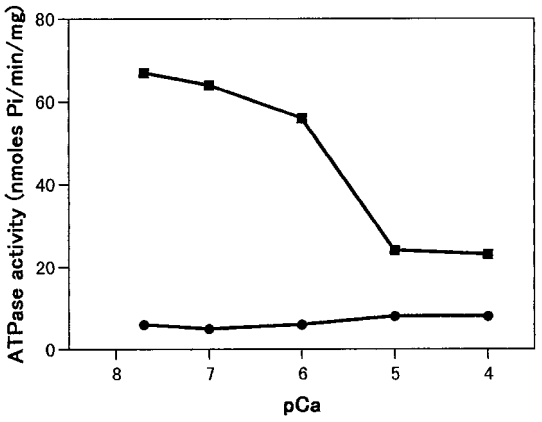

Figure 4. Effect of Ca^{2+} on the ATPase activity of 170-kD myosin in the presence (■) or absence (●) of F-actin. Average rates obtained from two separate preparations are shown.

arrowhead 2), were detected in addition to the 170-kD heavy chain (Fig. 5A, arrow, lane a). Since a large amount of casein, a 34-kD polypeptide, was supplemented in the homogenizing buffer as an antiproteolysis agent (Yokota and Shimmen, 1994), the 34-kD polypeptide is likely to be casein remaining in the 170-kD myosin fraction. The 18-kD polypeptide showed the same mobility in SDS-PAGE as that of spinach CaM (Fig. 5A, lane b). An antiserum against spinach CaM recognized the 18-kD component (Fig. 5B, lane A). This antiserum did not cross-react with the 170-kD heavy chain (Fig. 5B, lane a), whereas an antiserum against the 170-kD heavy chain recognized neither the 18-kD polypeptide nor spinach CaM (Fig. 5C).

In SDS-PAGE this 18-kD polypeptide exhibited a Ca^{2+}-dependent mobility shift, which is one of the characteristics of CaM (Burgess et al., 1980). The mobility of this peptide in 170-kD myosin pretreated with SDS-PAGE sample buffer supplemented with 2 mM $CaCl_2$ was faster than that in myosin pretreated with SDS-PAGE sample buffer supplemented with 1 mM EGTA (Fig. 6A). Furthermore, the mobility of this 18-kD peptide in the presence of $CaCl_2$ or EGTA was the same as that of spinach CaM in the presence of $CaCl_2$ or EGTA, respectively (Fig. 6). These results led us to the conclusion that the 18-kD polypeptide included in the 170-kD myosin fraction is CaM.

Interaction of CaM with the 170-kD Heavy Chain

To examine the association of CaM with the 170-kD heavy chain, an immunoprecipitation assay with an antiserum against the 170-kD heavy chain was carried out. As described above, this antiserum recognized only the 170-kD heavy chain, not CaM (Fig. 5C). Both the 170-kD heavy chain (Fig. 7A, lane a) and CaM (Fig. 7A, lane c) were immunoprecipitated with this antiserum. However, they were only faintly detected in the immunoprecipitate when the preimmune serum was used in the place of antiserum as a control (data not shown). The large band above the 45-kD molecular marker (Fig. 7A, lane c) corresponds to the rabbit IgG heavy chain in the serum.

It is interesting that CaM was not detected in the immunoprecipitate when the myosin fraction was pretreated with 1.5 mM $CaCl_2$ (Fig. 7A, lane d). The intensity of the 170-kD heavy-chain band did not change when pretreated with $CaCl_2$ (compare lanes a and b in Fig. 7A), indicating that Ca^{2+} does not inhibit the interaction of the 170-kD heavy chain with the antiserum. Therefore, it is suggested that CaM dissociates from the 170-kD heavy chain in the presence of high concentrations of Ca^{2+}. Because 1 mM EGTA was included in the myosin fraction, as described in "Materials and Methods," [Ca^{2+}] in the myosin fraction should increase to above 10^{-4} M by the addition of 1.5 mM $CaCl_2$.

Next, the threshold concentration of Ca^{2+} required for the dissociation of CaM from the 170-kD heavy chain was determined by the second procedure described in "Materials and Methods." The 170-kD myosin fraction was mixed with the antiserum against the 170-kD heavy chain in the presence of 1 mM EGTA, followed by the addition of protein A-conjugated beads. The 170-kD myosin bound to protein A beads through antibodies was treated with KEMP solution supplemented with Ca^{2+} of various concentrations (10^{-7} to 10^{-4} M). The bound material was then subjected to immunoblotting. CaM was recovered in the immunoprecipitate when the Ca^{2+} concentration in the KEMP solution was lower than 10^{-5} M (Fig. 7B, lanes a–d). In contrast, CaM was not detected when beads were treated with 10^{-4} M Ca^{2+} (Fig. 7B, lane e). The intensity of the 170-kD heavy-chain band in the immunoprecipitate was similar at all [Ca^{2+}] examined (data not shown).

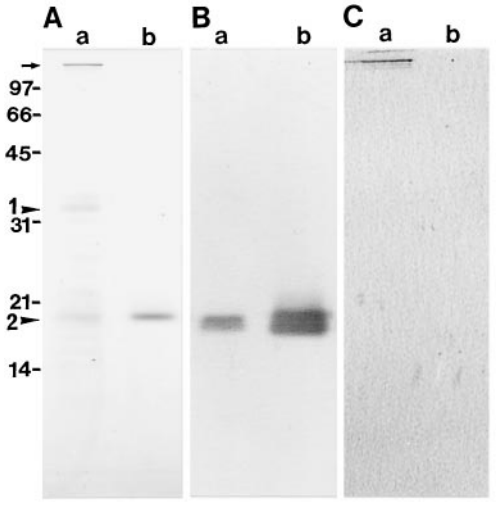

Figure 5. Immunoblotting of the 170-kD myosin fraction (a) and spinach CaM (b). The concentrations of 170-kD myosin and CaM applied on SDS-PAGE for each assay were 0.9 and 0.2 μg, respectively. A, Coomassie brilliant blue staining of a 15% polyacrylamide gel. B, Immunoblotting using antiserum against spinach CaM. C, Immunoblotting using antiserum against the 170-kD heavy chain. The arrow indicates the position of the 170-kD heavy chain. Arrowheads 1 and 2 indicate the 34- and the 18-kD polypeptide, respectively. The M_rs ($\times 10^{-3}$) of standard proteins are indicated on the left.

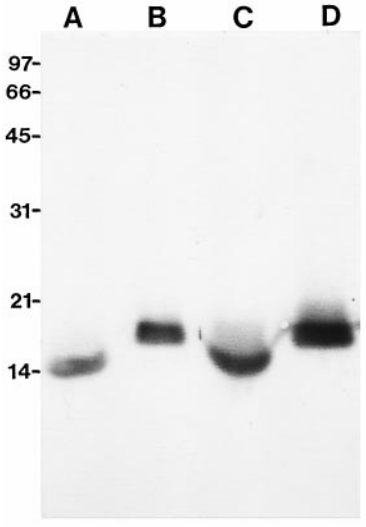

Figure 6. Electrophoretic mobility shift in SDS-PAGE by Ca^{2+}. Immunoblotting of 170-kD myosin (A and B) and spinach CaM (C and D) was carried out using antiserum against CaM. Each sample was treated with SDS-PAGE sample buffer supplemented with either 2 mM $CaCl_2$ (A and C) or 1 mM EGTA (B and D) and subjected to SDS-PAGE on a 15% polyacrylamide gel. M_rs ($\times 10^{-3}$) of standard proteins are indicated on the left.

Effect of Exogenous CaM on the Motile Inhibition by Ca^{2+}

The results presented above suggest that the motile activity of 170-kD myosin is inhibited irreversibly by treatment with 10^{-4} M Ca^{2+} because of the dissociation of CaM from the 170-kD heavy chain. Therefore, we examined the recovery effect of exogenously added CaM on the Ca^{2+}-inactivated 170-kD myosin. CaM was isolated from germinating lily pollen (Fig. 8A). The motile activity of 170-kD myosin remained suppressed after the following treatments: (a) A coverslip coated with 170-kD myosin was rinsed in a solution containing 10^{-4} M Ca^{2+} and then used for a motility assay in an assay medium containing 2 μM CaM and low concentrations of Ca^{2+} (10^{-6} and 10^{-7} M) or EGTA (data not shown). (b) The coverslip coated with 170-kD myosin was rinsed in a solution containing 10^{-4} M Ca^{2+} and 2 μM CaM and then used for a motility assay in an assay medium containing low concentrations of Ca^{2+} or EGTA (Fig. 8B, white bars). In contrast, when 2 μM CaM was added to both the rinsing solution and the assay medium, the percentage of translocated RP-labeled F-actin was recovered up to 45% to 60% (Fig. 8B, black bars). The sliding velocity of RP-labeled F-actin was also restored to between one-half and two-thirds of that induced by myosin that had not been rinsed with 10^{-4} M Ca^{2+} solution (data not shown).

DISCUSSION

This is the first report, to our knowledge, demonstrating that Ca^{2+} inhibits both the motile activity in vitro (Fig. 3) and the F-actin-stimulated ATPase activity (Fig. 4) in higher-plant myosin (170-kD myosin). It has been reported that the organelles isolated from lily pollen tubes are translocated along actin filaments in characean cells and that this translocation is inhibited by Ca^{2+}, indicating that myosin associated with organelles is equipped with a Ca^{2+}-sensitive mechanism (Kohno and Shimmen, 1988a). The [Ca^{2+}] required for this inhibition of organelle translocation along characean actin cables matches well with that required for the inhibition of 170-kD myosin (compare Fig. 3 in the present study with fig. 3 in Kohno and Shimmen [1988a]). These results, together with evidence that 170-kD myosin is associated with the membrane surface of organelles and that it is responsible for cytoplasmic streaming in lily pollen tubes (Yokota and Shimmen, 1994; Yokota et al., 1995a), suggest that Ca^{2+} sensitivity of 170-kD myosin may be a molecular basis for the Ca^{2+}-sensitive translocation of organelles.

In living lily pollen tubes, an intracellular Ca^{2+} gradient focused at the tip is present, and this is correlated with the tip growth (Nobiling and Reiss, 1987; Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992). In the tip region, which is referred to as the clear zone, active cytoplasmic streaming is not observed (Pierson et al., 1990, 1994; Lancelle and Hepler, 1992). Recently, Pierson et al. (1994, 1996) demonstrated that at the tip the [Ca^{2+}] is more than 3 μM (they considered 3–10 μM as a realistic range) and that it gradually decreases to a basal level of 0.2 μM within 20 μm from the pollen tube tip. When the [Ca^{2+}] at the tip is reduced by microinjection of Ca^{2+} buffer 1,2-bis-

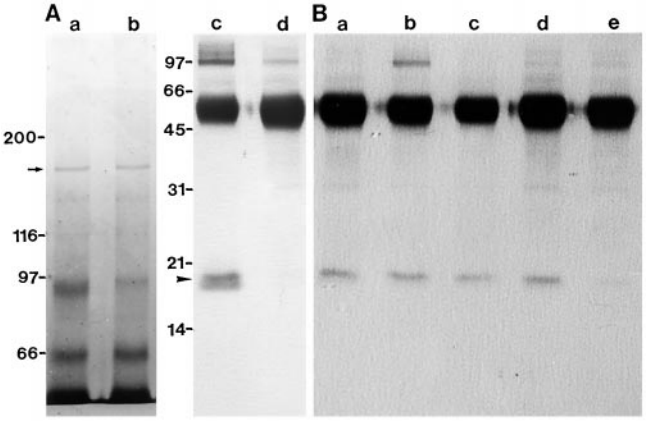

Figure 7. Immunoprecipitation of 170-kD myosin with antiserum against the 170-kD heavy chain. A, The 170-kD myosin was mixed with the antiserum without (a and c) or with (b and d) 1.5 mM $CaCl_2$ and subsequently mixed with protein A-conjugated beads. Specimens were centrifuged at 500*g* for 3 min. The materials bound to the beads were analyzed by SDS-PAGE on a 6% polyacrylamide gel (a and b) or by immunoblotting using antiserum against spinach CaM (c and d). B, The 170-kD myosin was mixed with antiserum against the 170-kD heavy chain without the addition of 1.5 mM $CaCl_2$ and subsequently mixed with protein A-conjugated beads. After the beads were washed with KEMP solution containing either EGTA (a) or Ca^{2+} at concentrations of 10^{-7} M (b), 10^{-6} M (c), 10^{-5} M (d), or 10^{-4} M (e), the 18-kD polypeptide associated with the beads was detected by the immunoblotting using the antiserum against spinach CaM. The arrow and arrowhead indicate the 170-kD heavy chain and the 18-kD polypeptide, respectively. M_rs ($\times 10^{-3}$) of standard proteins are indicated on the left.

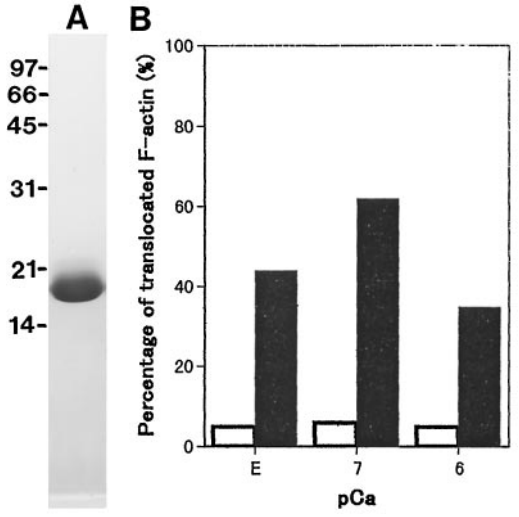

Figure 8. Purity of CaM isolated from lily pollen (A) and the effect of CaM on the motile activity of 170-kD myosin inactivated by 10^{-4} M Ca^{2+} (B). A, Coomassie brilliant blue staining of 15% polyacrylamide gel. B, Effect of CaM on the percentage of translocated RP-labeled F-actin. White bars, The coverslip coated with 170-kD myosin was rinsed by a solution containing 10^{-4} M Ca^{2+} and 2 μM CaM and then used for a motility assay in an assay medium containing Ca^{2+} at concentrations of 10^{-6} M (pCa 6), 10^{-7} M (pCa 7), or EGTA (E). Black bars, CaM (2 μM) was supplied to both the rinsing solution and the assay medium. M_rs ($\times 10^{-3}$) of standard proteins are indicated on the left in A.

(*o*-aminophenoxy)ethane *N*,*N*,*N*′,*N*′-tetraacetic acid or by a treatment with caffeine (Miller et al., 1992; Pierson et al., 1994, 1996), the elongation of lily pollen tubes is blocked and cytoplasmic streaming begins to be observed close to the tip. The Ca^{2+} inhibition of 170-kD myosin in vitro began at about 10^{-6} M and reached its plateau level at 2.5 μM (Fig. 3). This concentration range corresponds to that at the tip of elongating pollen tubes. In this [Ca^{2+}] range, the inhibition of 170-kD myosin was reversible (Fig. 3B). The 170-kD myosin is associated with organelles but it also exists at the tip, where active streaming is not observed (Yokota et al., 1995a). It is suggested that the reversible Ca^{2+} inhibition of 170-kD myosin is responsible for the absence of active streaming at the tip region.

It is well established that all myosins in nonplant cells whose primary structures have been determined contain at least one repeat of the IQ motif, which provides a binding site for CaM or a related protein of the EF-hand superfamily (Cheney and Mooseker, 1992; Mooseker and Cheney, 1995; Sellers et al., 1996). CaM has been shown to play a regulatory role in the activities of some of these myosins (Mooseker and Cheney, 1995; Wolenski, 1995). In plant myosin, heavy chains identified thus far by sequence analyses of their genes have several IQ motifs (Knight and Kendrick-Jones, 1993; Kinkema and Schiefelbein, 1994; Kinkema et al., 1994). However, no biochemical studies have been carried out to confirm whether CaM or a related protein is associated with the heavy chain as a light chain, since only a small number of plant myosins have been purified and characterized. In the present study we have shown the presence of an 18-kD polypeptide in the 170-kD myosin fraction (Fig. 5). Its molecular mass and mobility shift in SDS-PAGE, which are both dependent on Ca^{2+}, were similar to those of spinach CaM (Figs. 5 and 6). This polypeptide was recognized by an antiserum against spinach CaM (Fig. 5). Immunoprecipitation using an antiserum against the 170-kD heavy chain showed the association of the 18-kD polypeptide with the 170-kD heavy chain (Fig. 7). These results indicate unequivocally that the 18-kD polypeptide is CaM and that it is associated with the 170-kD myosin heavy chain as a light chain.

In characean cells it is hypothesized that the cessation of cytoplasmic streaming by Ca^{2+} is coupled to phosphorylation of myosin by a Ca^{2+}-dependent protein kinase (Tominaga et al., 1987). The activity of this protein kinase from soybean is enhanced by a several-micromolar concentration of Ca^{2+} (Harmon et al., 1987; Putnam-Evans et al., 1990). In the case of *Chara corallina*, isolated myosin did not show Ca^{2+} sensitivity for its motile and F-actin-stimulated ATPase activities (Yamamoto et al., 1994). This is one of the characteristics of myosin regulated by its phosphorylation (indirect myosin-linked Ca^{2+} regulation). In contrast, isolated pollen tube myosin (170-kD myosin) showed significant Ca^{2+}sensitivity (Figs. 3 and 4), suggesting that Ca^{2+} regulates myosin activity by binding to CaM, a light chain (direct myosin-linked Ca^{2+} regulation). It is likely that Ca^{2+} between 10^{-6} and 10^{-5} M causes reversible inhibition by an allosteric interaction of the 170-kD heavy chain and the CaM light chain. However, Ca^{2+} at concentrations higher than 10^{-5} M irreversibly inhibited the motility that was not reversible by Ca^{2+} removal (Fig. 3B).

Concomitantly, CaM was dissociated from the 170-kD heavy chain (Fig. 7B). In myosin I from the brush border of the intestine (Collins et al., 1990; Wolenski et al., 1993) or liver (Williams and Coluccio, 1994) or in myosin V from the brain (Cheney et al., 1993), Ca^{2+} modulates the motile activity of these myosins in vitro by binding to their CaM light chain. Ca^{2+} inhibits the motile activity of such myosins by partial dissociation of CaM from the heavy chain. This impaired activity is restored by exogenously supplied CaM. Also, in the case of 170-kD myosin under low concentrations of Ca^{2+}, the inactivated motility of 170-kD myosin was restored to some extent by exogenously supplied CaM that was isolated from lily pollen (Fig. 8B). However, it seems improbable that myosin encounters [Ca^{2+}] higher than 10^{-5} M in living pollen tubes.

Vahey et al. (1982) reported that the F-actin-stimulated ATPase activity of myosin isolated from tomato fruits is activated by increased Ca^{2+} concentrations. However, involvement of this myosin in cytoplasmic streaming seems unlikely, since several studies have shown that cytoplasmic streaming in somatic cells of higher plants is inhibited by Ca^{2+} (Shimmen and Yokota, 1994). We expect that our present results will provide a way to elucidate the molecular mechanism of the Ca^{2+} regulation of cytoplasmic streaming in higher plants.

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