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# Calcium-dependent regulation of the motor activity of recombinant full-length *Physarum* myosin

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**Ying Zhang[1,†], Hozumi Kawamichi[1,‡,†], Hideyuki Tanaka[2], Shinji Yoshiyama[1], Kazuhiro Kohama[1] and Akio Nakamura[1,*]**

[1]Department of Molecular and Cellular Pharmacology, Faculty of Medicine; and [2]Faculty of Health Sciences, Gunma University Graduate School of Medicine, 3-39-22 Showa-Machi, Maebashi, Gunma, Japan

*A. Nakamura, Department of Molecular and Cellular Pharmacology, Faculty of Medicine, Gunma University Graduate School of Medicine 3-39-22, Showa-Machi, Maebashi, Gunma 371-8511, Japan. Tel: +81-27-220-7962, Fax: +81-27-220-7966, email: gacho@med.gunma-u.ac.jp

[†]Y. Zhang and H. Kawamichi contributed equally to this work.
[‡]Present address: Hozumi Kawamichi, Department of Molecular Physiology and Medical Bioregulation, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Japan

**We successfully synthesized full-length and the mutant *Physarum* myosin and heavy meromyosin (HMM) constructs associated with *Physarum* regulatory light chain and essential light chain (PhELC) using *Physarum* myosin heavy chain in Sf-9 cells, and examined their $Ca^{2+}$-mediated regulation. $Ca^{2+}$ inhibited the motility and ATPase activities of *Physarum* myosin and HMM. The $Ca^{2+}$ effect is also reversible at the *in vitro* motility of *Physarum* myosin. We demonstrated that full-length myosin increases the $Ca^{2+}$ inhibition more effectively than HMM. Furthermore, $Ca^{2+}$ did not affect the motility and ATPase activities of the mutant *Physarum* myosin with PhELC that lost $Ca^{2+}$-binding ability. Therefore, we conclude that PhELC plays a critical role in $Ca^{2+}$-dependent regulation of *Physarum* myosin.**

*Keywords*: Actin/ATPase activity/calcium/in vitro motility assay/*Physarum* myosin.

*Abbreviations*: HC, heavy chain; HMM, heavy meromyosin; LC, light chain; Ph·HC, *Physarum* myosin heavy chain; PhELC, *Physarum* essential light chain; PhRLC, *Physarum* regulatory light chain.

The plasmodium of *Physarum polycephalum* exhibits contraction and relaxation movement associating periodic cycles of cytoplasmic streaming. The periodic changes of intracellular $Ca^{2+}$ ($[Ca^{2+}]i$) have been known for many years and is related with the movement; the increase in $[Ca^{2+}]i$ is a signal for relaxation and the decrease in $[Ca^{2+}]i$ for contraction (*1*). This effect of $[Ca^{2+}]i$ was different from the regulatory mode muscle contraction and regulation (*2*). Our recent study with the living plasmodium was in conformity with the unprecedented regulatory mode (*3*). Most myosins that belong to myosin II family are unable to bind $Ca^{2+}$ with high affinity enough to regulate the actin-myosin interaction. However, the interaction between actin and myosin purified from *Physarum* was induced without requiring $Ca^{2+}$, and $Ca^{2+}$ worked so as to inhibit the interaction (*4*). Subsequently, *Physarum* myosin, myosin II isoform was purified; the myosin bound $Ca^{2+}$, and its actin-activated ATPase was inhibited by $Ca^{2+}$ (*5*).

On the other hand, the myosins from scallop muscle (*6*) and from *Physarum* (*5*) bind $Ca^{2+}$ to regulate the interaction. The effect of $Ca^{2+}$ on the former is activating the interaction, and that on the latter is inhibiting the interaction (*5*, *6*). In spite of the diametrically different effect, both myosins are composed of heavy chain (HC) and a pairs of regulatory light chain (RLC) class and essential light chain (ELC) class (*7*–*9*). To see the role of RLCs and ELCs, the hybrid myosins of smooth muscle HC, associating ELCs and RLCs from *Physarum*/scallop myosins, were expressed followed by the purification of the recombinant proteins. The myosin showed that the inhibiting effect and the activating effect were attributed to *Physarum* ELC (PhELC) and to scallop ELC (ScELC), respectively (*10*). However, the problem of this study is that HC was from smooth muscle origin. Obviously, the HCs of *Physarum* and scallop myosins should be expressed for the establishing the role of ELC. In this study, we succeeded in expressing *Physarum*, not scallop, myosin with *Physarum* HC at full-length associating PhELC and PhRLC as follows later in the text. We also obtained the mutant *Physarum* myosin with PhELC that lost $Ca^{2+}$-binding ability. Furthermore, we measured the effect of $Ca^{2+}$ on the motility and ATPase activities of *Physarum* myosin and its mutant.

## Materials and Methods

### Materials

ATP was purchased from Sigma-Aldrichi Chemical Corp. (St. Louis, MO, USA). Dithiothreitol (DTT) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (ABSF) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Glucose oxidase and catalase were purchased from Sigma. Restriction and other modifying enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan). All other chemicals were commercial products of reagent grade. Solutions were made using MilliQ water (Millipore, MA, USA). Actin was purified from the acetone powder of the

chicken skeletal muscle by the method of Spudich and Watt (*11*), with slight modification (*12*), and used as actin filaments after polymerization.

### Construction of recombinant baculovirus transfer vectors

We constructed a recombinant baculovirus by using the Bac-to-Bac system (Invitrogen) with the genes for Ph·HC (GenBank$^{TM}$ accession number AF335500), PhRLC (GenBank$^{TM}$ accession number AB076705) and PhELC (GenBank$^{TM}$ accession number J03499), according to the Invitrogen protocol. We have already reported that the triple mutant of D15A/D17A/E26A in EF-hand I motif of PhELC completely abolished $Ca^{2+}$ binding to ELC by $Ca^{2+}$ binding experiments (*13*, *14*). According to the previous report, we have constructed PhELC containing three point mutations of D15A/D17A/E26A in EF-hand I motif. PhELC that lost in $Ca^{2+}$-binding activity was confirmed by use of $Ca^{2+}$ binding assay as described earlier (*13*). The mutant was named PhELC-3A as described in our previous report. The report also described about the recombinant baculovirus *Physarum* heavy meromyosin (HMM) (PhHMM·HC) and *Physarum* HMM with PhELC-3A (*13*). To facilitate the purification of *Physarum* myosin, a His-tag sequence was further attached to the C-terminus of cDNA of Ph·HC. The details of construction of recombinant baculovirus is described as previously reported (*10*).

### Expression and purification of recombinant Physarum myosin and its mutant

The Sf9 cells were co-infected with three separate viruses (Ph·HC, PhRLC and PhELC or PhELC-3A) at the same multiplicity of infection of 1. The infected cells were grown for 72 h at 27°C. The cells expressing a *Physarum* myosin were then pelleted by centrifugation, followed by suspension in buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptethanol, 1 mM ABSF and His-Tag protein protease inhibitor cocktail (Roche, IN, USA). The lysis was adjusted to 0.2 M NaCl and 1 mM ATP after sonication to release recombinant myosin from endogenous actin and then centrifuged for 1 h at 15,000 × g to remove cell debris and unbroken cells. The supernatant was mixed with Ni-NTA agarose (Qiagen, Germany) in a 50 ml beaker with a rotating wheel for 3 h at 4°C and then loaded on a column. The column was washed with binding buffer containing of 0.6 M NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 20 mM imidazole (pH 7.5), 0.1 mM ABSF and 5 mM 2-mercaptethanol. The *Physarum* myosin was then eluted with elution buffer containing of 0.6 M NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 200 mM imidazole (pH 7.5), 0.1 mM ABSF and 5 mM 2-mercaptethanol. Fractions were subjected to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and those containing myosin were pooled and concentrated to 500 µl using an Amicon Ultra centrifugal Filter Unit (Millipore, MA, USA). The concentrate was further subjected to Superose$^{TM6}$ column chromatography (GE Healthcare, Buckingham, UK) in 0.3 M KCl, 20 mM Tris-HCl (pH 7.5) and 5 mM 2-mercaptethanol. The eluent from the column containing myosin was concentrated to 0.05–0.1 ml using the filter unit. With this procedure, approximately 100 µg myosin was obtained from $10^8$ cells. A similar procedure was used for purification of mutant *Physarum* myosin-3A and HMM. All the protein samples were used within 1 week and were always kept on ice.

### Electron microscopy

Purified *Physarum* myosin, HMM and the mutant *Physarum* myosin-3A were visualized by the rotary shadow electron microscope method as described previously (*15*). Briefly, purified *Physarum* myosin, HMM or *Physarum* myosin-3A (0.05 mg/ml) was attached to a freshly cleaved mica sheet. The mica sheet was washed three times with 30% glycerol in 0.1 M ammonium acetate, stained with 2% aqueous uranylacetate and then washed three times. The mica sheet was covered, pushed and peeled off with another cleaved mica sheet and then rotary replicated with platinum at low angles in a BAF 060 rotary shadowing system. The replicated specimen was placed on a specimen grid and observed with a JEM-1010 electron microscope.

### ATPase assay

We measured the ATPase activity (specific activity of *Physarum* myosin as expressed by $s^{-1}$ per myosin head) by continuously measuring the release of inorganic phosphate (Pi), according to the method of Webb (*16*), using an EnzChek Phosphate Assay Kit (Invitrogen, CA, USA). The ATPase activity was measured in the reaction mixture containning 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM DTT, 0.5 mM ATP, 0.05 µM *Physarum* myosin, 5 µM actin filaments as expressed by monomeric actin concentration and 0.1 mM EGTA or the different concentration of $Ca^{2+}$ (pCa = 8, 7, 6, 5 and 4). After pre-incubating for 10 min at 25°C, the reaction was started by adding ATP to monitor the released Pi.

### In vitro motility assay

The *in vitro* motility assay was performed as described previously (*17*) with slight modifications (*18*). Briefly, actin filaments labeled with rhodamine-phalloidin (Molecular Probes, USA) were introduced into the flow cells constructed between a glass slide and a coverslip coated with *Physarum* myosin in motility assay buffer consisting of 10 mM KCl, 2 mM ATP, 1 mM $MgCl_2$, 10 mM imidazole (pH 7.5), 25 mM DTT and 0.1 mM EGTA or the different concentration of $Ca^{2+}$ (pCa = 8, 7, 6, 5 and 4). Glucose oxidase (0.2 mg/ml), catalase (0.04 mg/ml) and glucose (4.5 mg/ml) were added to the motility assay buffer to prevent photobleaching of the rhodamine. To detect the reversibility of the effect, $Ca^{2+}$ that remained in the flow cell was washed out three times with motility buffer, and the flow cell was perfused with F-actin in the motility buffer in 0.1 mM EGTA. The movement of actin filaments was recorded under a fluorescence microscope equipped with a silicone-intensifier target camera. The average velocity was determined using

WCIF Image J. The experiment results were presented as the mean ± SD of 60 actin filaments for each experiment.

### Other procedures

SDS-PAGE was carried out on a 12.5% polyacrylamide gel using the buffer system of Laemmli (*19*) with slight modification (*20*). Protein concentrations were determined by Bio-Rad protein assay (*21*) using bovine serum albumin as a standard.

## Results

### Expression and purification of Physarum myosin, HMM and mutant

Sf-9 cells were co-infected with genes for HCs and LCs as previously described (*10*). After the co-infected cells were cultured for 72 h, we harvested the cells and purified the proteins according to the methods described by Zhang *et al.* (*10*). Fig. 1A presents a schematic diagram of *Physarum* myosin. *Physarum* myosin at full-length comprises Ph·HC in association with PhELC and PhRLC (*22*). SDS-PAGE of this purified *Physarum* myosin is shown in lane 1 of Fig. 1B. In addition, we obtained the mutant *Physarum* myosin-3A, comprising Ph·HC associated with PhELC-3A and PhRLC (Fig. 1B, lane 2) and *Physarum* HMM, comprising PhHMM·HC associated with PhELC and PhRLC (Fig. 1B, lane 3). Each *Physarum* myosin construct contained HC, PhELC and PhRLC with a stoichiometry of 1.0:0.9–1.1:0.9–1.0, as determined by gel densitometry (data not shown).

To visualize the nature of purified *Physarum* myosin, HMM and the mutant *Physarum* myosin-3A, these samples were absorbed to fleshly cleaved mica and then subject to rotary shadowing as described previously (*15*). The electron micrograph of *Physarum* myosin indicates that its structure comprises two globular heads and a long tail irrespective of mutation in PhELC (Fig. 2A and 2B). The HMM nature was confirmed by the short tail (Fig. 2C).

### The effect of $Ca^{2+}$ on motor activity of Physarum myosin, HMM and mutant

The $Ca^{2+}$-dependent regulation of the motor activity of the expressed *Physarum* myosin constructs were examined using an *in vitro* motility assay. We coated a glass surface with the *Physarum* myosin. The velocity of actin filaments in the presence of *Physarum* myosin in the presence of 0.1 mM EGTA and 0.1 mM $Ca^{2+}$ (pCa = 4) was $0.995 \pm 0.204$ and $0.234 \pm 0.078$ μm/s, respectively. In addition, the movement of actin filaments on the glass coated by *Physarum* HMM was also decreased by $Ca^{2+}$. In the presence of 0.1 mM EGTA and *Physarum* HMM, actin filaments moved at a velocity of $0.754 \pm 0.134$ μm/s; in the presence of 0.1 mM $Ca^{2+}$ (pCa = 4) and *Physarum* HMM, actin filaments moved at a velocity of $0.450 \pm 0.104$ μm/s. Moreover, these inhibitory effects were $Ca^{2+}$ concentration dependent. The inhibition of actin filament velocity increased as the concentration of $Ca^{2+}$ increased (Fig. 3A and 3B). These observations were consistent with those for the myosin (*23*). However, the movements of actin filaments in the presence of the mutant *Physarum* myosin where PhELC replaced by PhELC-3A were not altered, irrespective of the presence of 0.1 mM EGTA or 0.1 mM $Ca^{2+}$ (pCa = 4) (Fig. 3A).

### Reversibility of the $Ca^{2+}$ effect on Physarum myosin

Reversibility of the $Ca^{2+}$ effect will be the crucial test as to whether myosin is native, as indicated in Fig. 7 of ref. *4* using the purified *Physarum* myosin as shown in Fig. 4. We changed the motility buffer in the order EGTA→$Ca^{2+}$→EGTA and observed the movement of actin filaments in the presence of *Physarum* myosin on the same coverslip. We observed that $Ca^{2+}$ inhibited the movement of actin filaments, when the motility buffer was changed from EGTA to $Ca^{2+}$ (Fig. 4A). Then, on changing the buffer from $Ca^{2+}$ to EGTA, we observed that the movement of actin filaments recovered. Our results showed that the movements of actin filaments on *Physarum* myosin were slow in the $Ca^{2+}$ buffer, whereas the movements became faster when the buffer was changed from $Ca^{2+}$ to EGTA (Fig. 4B).

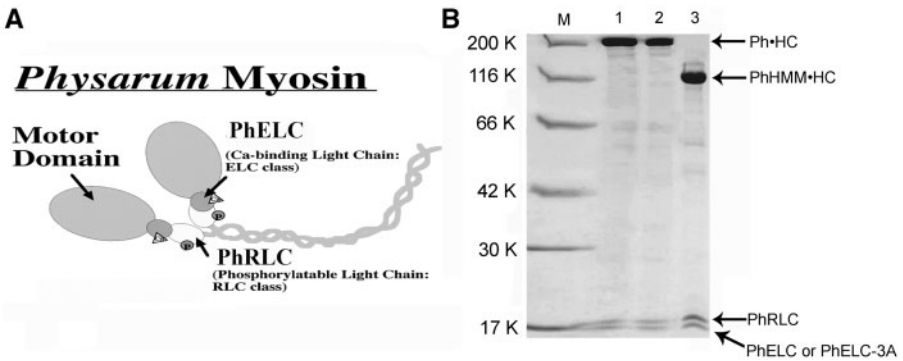


**Fig. 1 Schematic diagram and purification of *Physarum* myosin constructs.** (**A**) Schematic diagram of *Physarum* myosin comprising Ph·HC associated with PhRLC and PhELC. The PhRLC and PhELC were localized according to ref. 12. (**B**) 12.5% SDS-PAGE of purified *Physarum* myosin, myosin-3A and HMM associated with LCs of *Physarum* myosin. Lane M: Molecular weight marker. Lane 1: *Physarum* myosin; lane 2: *Physarum* myosin-3A; lane 3: *Physarum* HMM.

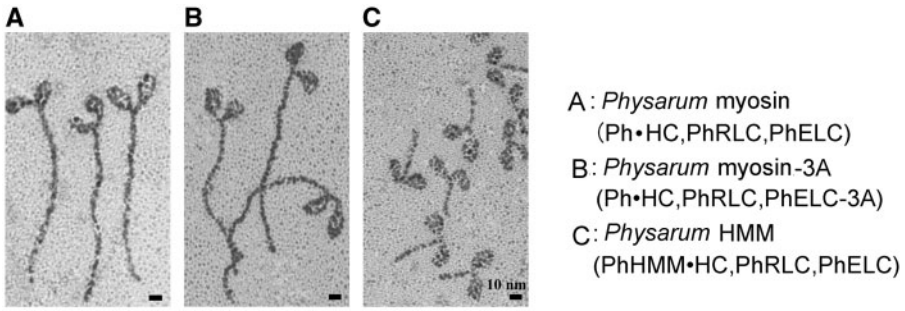


**Fig. 2 Electron micrographs of the *Physarum* myosin constructs.** Purified *Physarum* myosin constructs were visualized by rotary shadow-casting electron microscopy. The head and tail structures were shown in three *Physarum* myosin constructs (scale bar, 10 nm). A: *Physarum* myosin; B: *Physarum* myosin-3A; C: *Physarum* HMM.

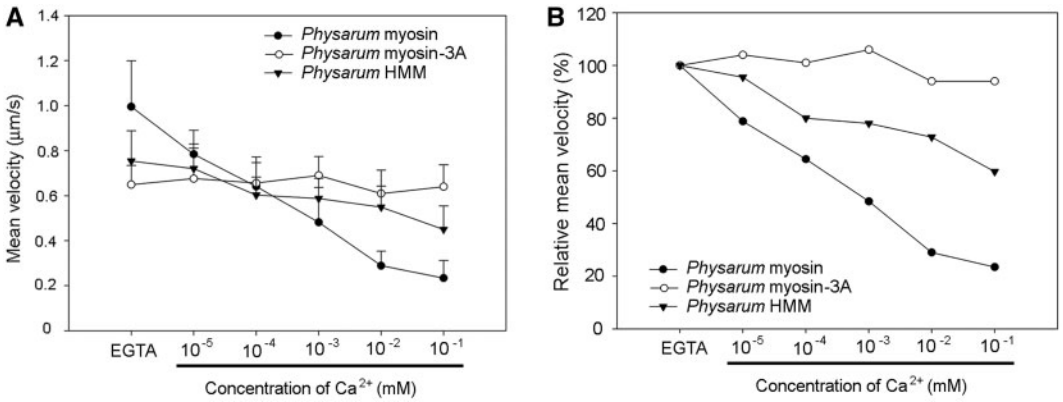


**Fig. 3 Effects of $Ca^{2+}$ on the movement velocity of actin filaments on *Physarum* myosin constructs.** (**A**) Velocity of actin filament in the presence of *Physarum* myosin, myosin-3A and HMM in EGTA and at different concentrations of $Ca^{2+}$. Values are mean ± SD, $n = 60$. (**B**) Relative effect of $Ca^{2+}$ on *Physarum* myosin, myosin-3A and HMM.

### *The effect of $Ca^{2+}$ on ATPase activities of Physarum myosin, HMM and mutant*

Then, we measured the actin-activated ATPase activities of the *Physarum* myosin constructs in the presence and absence of $Ca^{2+}$. The ATPase activity of *Physarum* myosin was reduced from $1.382 \pm 0.142\ s^{-1}$ in 0.1 mM EGTA to $0.046 \pm 0.016\ s^{-1}$ in 0.1 mM $Ca^{2+}$ (pCa = 4). Similarly, the ATPase activity of *Physarum* HMM was reduced from $0.889 \pm 0.072\ s^{-1}$ in 0.1 mM EGTA to $0.503 \pm 0.017\ s^{-1}$ in 0.1 mM $Ca^{2+}$ (pCa = 4). However, $Ca^{2+}$ did not inhibit the ATPase activity of the *Physarum* myosin-3A even in 0.1 mM $Ca^{2+}$ (pCa = 4) (Fig. 5A).

## Discussion

In the present study, we successfully obtained recombinant full-length *Physarum* myosin in Sf-9 cells. The length of the myosin tail expressed by baclovirus expression system is about 160 nm and confirms its full-length nature comparing that of the myosin purified from *Physarum* plasmodium (*5*). We demonstrated that $Ca^{2+}$-dependent regulation of the motor activity and ATPase activity of *Physarum* myosin and HMM. However, we did not observe the effects of $Ca^{2+}$ on the motility and ATPase activities of the mutant with PhELC of three mutating acidic residues at D15A, D17A and E26A. Our results suggest that PhELC plays an important role on $Ca^{2+}$-dependent regulation of *Physarum* myosin.

As the $Ca^{2+}$-binding sites of *Physarum* myosin are located at EF-hand I motif of PhELC (*13*, *14*), to confirm the $Ca^{2+}$-dependent regulation of *Physarum* myosin, we performed the loss-of-function studies. We constructed the mutant PhELC-3A by mutation of three amino acidic residues in EF-hand I motif and obtained the mutant *Physarum* myosin-3A. We also confirmed the complete loss of $Ca^{2+}$-binding ability of PhELC-3A and mutant *Physarum* myosin-3A by using of $Ca^{2+}$-binding assay (data not shown). $Ca^{2+}$ inhibited the motility and ATPase activities of *Physarum* myosin (Figs. 3 and 5). However, $Ca^{2+}$ did not affect the motility and ATPase activities of the mutant *Physarum* myosin-3A (Figs. 3 and 5). These results suggest that *Physarum* myosin is a $Ca^{2+}$-dependent regulatory molecule.

According to the ATPase activity results, full-length *Physarum* myosin exhibited higher ATPase activity than the mutant *Physarum* myosin-3A and HMM constructs in 0.1 mM EGTA (Fig. 5A). This is consistent with the results of the *in vitro* motility assay. The movements of actin filaments on the full-length *Physarum* myosin ($0.995 \pm 0.204$ μm/s) were faster than that of HMM ($0.754 \pm 0.134$ μm/s) and the mutant *Physarum* myosin-3A ($0.649 \pm 0.120$ μm/s). On the other hand, we observed that the effect of $Ca^{2+}$ on

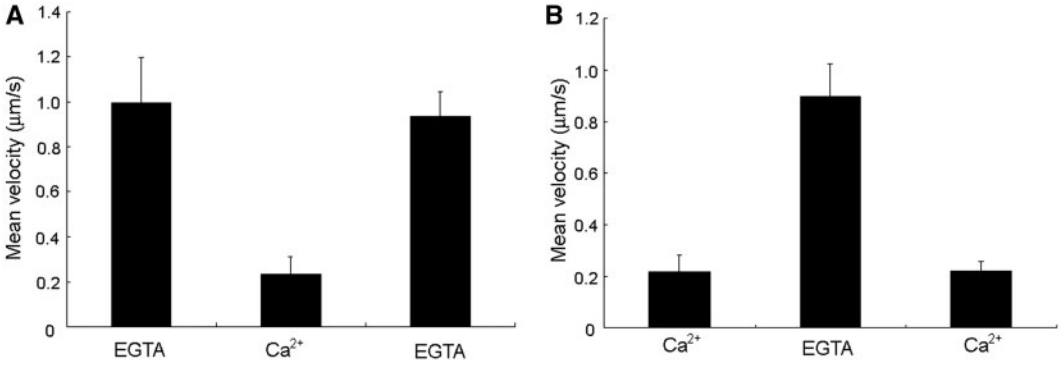


**Fig. 4 Reversibility of the $Ca^{2+}$ effect on velocity of actin filaments on *Physarum* myosin in EGTA and in $Ca^{2+}$.** (**A**) The velocity of actin filaments on *Physarum* myosin in the motility buffer in the order EGTA→$Ca^{2+}$→EGTA. (**B**) The velocity of actin filaments on *Physarum* myosin in the motility buffer in the order $Ca^{2+}$→EGTA→$Ca^{2+}$. Values are mean ± SD, $n = 60$.

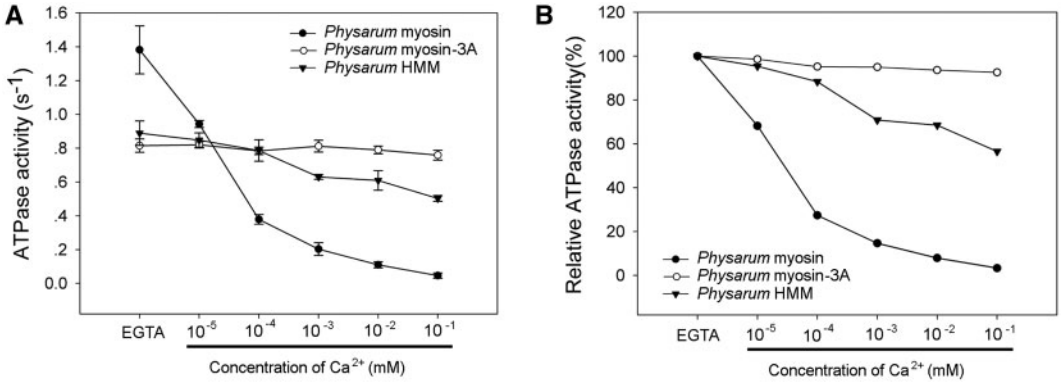


**Fig. 5 Specific actin-activated ATPase activities of *Physarum* myosin constructs.** (**A**) ATPase activity was measured in the presence of *Physarum* myosin, myosin-3A and HMM in EGTA and at different concentrations of $Ca^{2+}$. Values are mean ± SD, $n = 5$. (**B**) Relative effect of $Ca^{2+}$ on *Physarum* myosin, myosin-3A and HMM.

ATPase activities of the constructs was of myosin > HMM > myosin-3A (Fig. 5B). These observations were consistent with the findings for the movement of actin filaments on the corresponding *Physarum* myosin constructs in the *in vitro* motility assay. The velocity of actin filaments was of myosin > HMM > myosin-3A in 0.1 mM EGTA (Fig. 3A). The velocities of these constructs were not changed in pCa = 7. Moreover, we observed that the ATPase activity of the full-length *Physarum* myosin remarkably changed in the presence of 0.1 mM $Ca^{2+}$ compared with that observed in the presence of 0.1 mM EGTA (96.67% inhibition). However, the ATPase activity of *Physarum* myosin-3A was hardly changed in the presence of 0.1 mM $Ca^{2+}$ compared with that in the presence of 0.1 mM EGTA (Fig. 5B). Similar effect of $Ca^{2+}$ was also confirmed by the significant inhibition of actin filament movement along *Physarum* myosin, but not along mutant *Physarum* myosin-3A, in the presence of $Ca^{2+}$ (Fig. 3B). According to the findings that $Ca^{2+}$ could not influence the movements of actin filaments and the ATPase activity of *Physarum* myosin when three amino acids of PhELC were mutated to alanine, we concluded that PhELC might determine the effect of $Ca^{2+}$ on *Physarum* myosin. Taken together, these results suggested that PhELC plays an important role in the inhibitory effects of $Ca^{2+}$-dependent regulation of *Physarum* myosin.

On the other hand, we detected significantly the inhibitory effects of $Ca^{2+}$ on the full-length *Physarum* myosin compared with that on *Physarum* HMM. Previous report by Kawamichi *et al* (*13*) has also been reported that $Ca^{2+}$ slightly affected actin-dependent ATPase activity using recombinant *Physarum* HMM with PhELC and PhRLC. Comparing their structures between full-length *Physarum* myosin and *Physarum* HMM, full-length *Physarum* myosin has a different structure of long tail (Fig. 2). The recent evidences demonstrate that interactions between the heads and the distal tail perform a critical role in regulating activity of myosin II molecules through stabilizing the compact monomer conformation (*24*). Therefore, we speculate that the long tail may help to inhibitory effects of $Ca^{2+}$ by the interactions with the heads.

In addition, we attempted to obtain a *Physarum* myosin hybrid with LCs from the myosins of *Physarum* and scallop. We measured the effect of $Ca^{2+}$ on the ATPase activity of the *Physarum* myosin hybrids. The ATPase activity of *Physarum* myosin hybrid of Ph·HC with PhELC and ScRLC was reduced from $0.592 \pm 0.050\ s^{-1}$ in 0.1 mM EGTA to $0.119 \pm 0.029\ s^{-1}$

in 0.1 mM $Ca^{2+}$ (pCa = 4). On the other hand, the ATPase activity of *Physarum* myosin hybrid of Ph·HC with PhRLC and ScELC was reduced from $0.628 \pm 0.016\,s^{-1}$ in 0.1 mM EGTA to $0.378 \pm 0.008\,s^{-1}$ in 0.1 mM $Ca^{2+}$ (pCa = 4). However, we did not observe the movements of actin filaments along these constructs (data not shown). Gourinath *et al*. reported that conformational differences exist in the crystal structures at the motor domain-lever arm junction between scallop and vertebrate smooth muscle myosins (*9*). Therefore, we hypothesize that conformational differences exist between scallop and *Physarum* myosin. More details about the crystal structure are required to clarify the communication between HC/LCs and two pairs of LCs and the intramolecular signal transmission in $Ca^{2+}$ inhibition of *Physarum* myosin.

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### Conflict of interest

None declared.

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