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Original Article

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Calcium-mediated regulation of recombinant hybrids of full-length *Physarum* myosin heavy chain with *Physarum*/scallop myosin light chains

Ying Zhang[1], Hozumi Kawamichi[2], Kazuhiro Kohama[3], and Akio Nakamura[2,*]

[1]Department of Molecular Physiology and Medical Bioregulation, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan, [2]Department of Molecular Pharmacology and Oncology, Faculty of Medicine, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan, and [3]Research Institute of Pharmaceutical Sciences, Musashino University, Nishitokyo, Tokyo 202-8585, Japan

*Correspondence address. Tel: +81-27-220-7962; Fax: +81-27-220-7963; E-mail: gacho@gunma-u.ac.jp

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Abstract

Physarum myosin is a Ca^{2+}-binding protein and its activity is inhibited by Ca^{2+}. In the present study, to clarify the light chains (LCs) from the different species (*Physarum* and scallop) and to determine the specific Ca^{2+}-regulated effects, we constructed hybrid myosins with a *Physarum* myosin heavy chain (Ph·HC) and *Physarum* and/or scallop myosin LCs, and examined Ca^{2+}-mediated regulation of ATPases and motor activities. In these experiments, it was found that Ca^{2+} inhibited motilities and ATPase activities of *Physarum* hybrid myosin with scallop regulatory light chain (ScRLC) and *Physarum* essential light chain (PhELC) but could not inhibit those of the *Physarum* hybrid myosin mutant Ph·HC/ScRLC/PhELC-3A which lacks Ca^{2+}-binding ability, indicating that PhELC plays a critical role in Ca^{2+}-mediated regulation of *Physarum* myosin. Furthermore, the effects of Ca^{2+} on ATPase activities of *Physarum* myosin constructs are in the following order: Ph·HC/PhRLC/PhELC > Ph·HC/ScRLC/PhELC > Ph·HC/PhRLC/ScELC > Ph·HC/ScRLC/ScELC, suggesting that the presence of PhRLC and PhELC leads to the greatest Ca^{2+} sensitivity of *Physarum* myosin. Although we did not observe the motilities of *Physarum* hybrid myosin Ph·HC/PhRLC/ScELC and Ph·HC/ScRLC/ScELC, our results suggest that Ca^{2+}-binding to the PhELC may alter the flexibility of the regulatory domain and induce a 'closed' state, which may consequently prevent full activity and force generation.

Key words: actin, ATPase activity, calcium, *in vitro* motility assay, *Physarum* hybrid myosin

Introduction

Myosins constitute a superfamily of motor proteins that play important roles in several cellular processes that produce force, translocation, and muscle contraction [1–4]. Regulation via myosin follows either Ca^{2+}-dependent phosphorylation of myosin regulatory light chains (RLCs) or direct binding of Ca^{2+} to essential light chains (ELCs). The former mode of regulation occurs in vertebrate smooth muscle, and it is widely accepted that contraction of smooth muscle is initiated by increases in intracellular Ca^{2+} concentrations. Specifically, Ca^{2+}–calmodulin binding results in a complex that binds and activates myosin light chain kinase (MLCK). Subsequently, activated MLCK phosphorylates myosin RLCs to induce the contraction of

smooth muscle [5,6]. The latter mechanism involving direct Ca^{2+}-binding has been shown in some molluscan muscles [7,8] and in lower eukaryotes [9,10]. Mollusk scallop myosin belongs to the myosin II family, and its activity is regulated by Ca^{2+}-binding to ELC [11]. Moreover, a similar myosin II family member from *Physarum polycephalum* is also regulated by Ca^{2+}-binding to ELC [12]. However, in contrast to scallop myosin, the activity of *Physarum* myosin is inhibited by Ca^{2+} [13]. Despite these diametrically opposing effects, both of these myosins comprise heavy chain (HC) and pairs of RLC class and ELC class molecules that bind Ca^{2+} [14]. Several previous studies have demonstrated the Ca^{2+}-activating regulation of scallop myosin [15,16]. However, the mechanism by which Ca^{2+} inhibits the activity of *Physarum* myosin remains poorly characterized.

Previously, we constructed hybrid heavy meromyosin (HMM) of smooth muscle HMM-HC associated with ELCs and RLCs from *Physarum*/scallop myosins, and purified recombinant hybrid smooth muscle HMMs following expression in Sf-9 cells. The ATPase assay and *in vitro* motility assay demonstrated that the inhibiting and activating effects were related to *Physarum* ELC (PhELC) and scallop ELC (ScELC), respectively [14]. Subsequently, recombinant full-length *Physarum* myosin was obtained and the Ca^{2+}-mediated regulation was identified [12]. However, whether light chains (LCs) from different species (*Physarum* and scallop) show specific Ca^{2+}-regulated effects or not still remains unclear. Here, we further examine the roles of LCs from *Physarum* and scallop to assess the role of LCs and examine the interactions between RLC and ELC.

We expressed hybrid myosins of *Physarum*, including myosin with full-length *Physarum* HC (Ph·HC) associated with *Physarum* light chains (PhRLC and PhELC), and with scallop light chains (ScRLC and ScELC). We also generated a hybrid mutant *Physarum* myosin with ScRLC and PhELC-3A and showed loss of Ca^{2+}-mediated regulation. Finally, we measured the effect of Ca^{2+} on motilities and the ATPase activities of hybrid *Physarum* myosins and mutants.

Materials and Methods

Materials

ATP was purchased from Sigma-Aldrich (St Louis, 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-Aldrich. Restriction and other modifying enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan). All other chemicals were commercial products of reagent grade. Solutions were prepared using MilliQ water (Millipore, Billerica, USA).

Actin was purified from the acetone powder of chicken skeletal muscle according to Spudich and Watt [17] with slight modifications and used as actin filaments after polymerization.

Construction of recombinant baculovirus transfer vectors

Recombinant baculovirus vectors were constructed using the Bac-to-Bac system (Invitrogen, Carlsbad, USA) with genes for Ph·HC (GenBank™ accession number AF335500), PhRLC (GenBank™ accession number AB076705), ScRLC (GenBank™ accession number M17208), PhELC (GenBank™ accession number J03499), and ScELC (GenBank™ accession number M17201) according to the manufacturer's protocol. PhELC with no Ca^{2+}-binding activity was generated by substituting D15, D17, and E26 positions with alanines and was designated PhELC-3A as described in our previous report [12]. To facilitate purification of *Physarum* myosin, a His-tag sequence was attached to the C-terminus of Ph·HC cDNA based on our previous report [14]. Recombinant baculovirus constructs were generated as previously reported [12,14].

Expression and purification of recombinant hybrid myosins

Recombinant hybrid myosins were expressed and purified as described previously [12,14]. Briefly, Sf-9 cells were co-infected with three separate viruses (HC, RLC, and ELC) at a multiplicity of infection of 1. Infected cells were grown for 72 h at 27°C and those expressing *Physarum* hybrid myosins were pelleted by centrifugation and resuspended in buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 5 mM 2-mercaptethanol, 1 mM ABSF, and His-tag protein protease inhibitor cocktail (Roche, Indianapolis, USA). To release recombinant myosin from endogenous actin, the lysis buffer was adjusted to 0.2 M NaCl and 1 mM ATP after sonication, and lysates were then centrifuged for 1 h at 15,000 *g* to remove cell debris and unbroken cells. Subsequently, supernatants were mixed with Ni-nitrilotriacetic acid agarose affinity column chromatography solution (Qiagen, Hilden, Germany) in a 50-ml beaker with a rotating wheel for 3 h at 4°C and were then loaded onto a column. The column was washed with binding buffer containing 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, and *Physarum* hybrid myosin was eluted with buffer containing 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 subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and those containing myosin were pooled and concentrated into 500 µl using an Amicon Ultra centrifugal Filter Unit (Millipore). Concentrates were then subject to Superose^{TM6} column chromatography (GE Healthcare, Buckingham, UK), and eluents containing myosin were concentrated into 0.05–0.1 ml using the filter unit. Approximately 100 µg of myosin was obtained from 1×10^8 cells using this procedure. All protein samples were used within 1 week and were kept on ice throughout the procedures.

ATPase assay

ATPase activities (specific activity of *Physarum* myosin, s^{-1} per myosin head) were determined by continuous measurements of inorganic phosphate (Pi) release using an EnzChek Phosphate Assay Kit (Invitrogen) according to the method of Webb as described in our previous report [18]. Briefly, ATPase activity was measured in reaction mixtures containing 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, and 0.1 mM EGTA or various concentrations of Ca^{2+} (pCa: 8, 7, 6, 5, and 4) obtained by using Calcon 3.6 software to calculate the amount of Ca^{2+} (**Supplementary Table S1**). After 10 min of pre-incubation at 25°C, reactions were initiated by adding ATP, and Pi release was monitored for 5 min. It must be noted that our results may not reflect the changes in maximal ATPase activity since we did not do ATPase measurements at saturating actin concentrations.

In vitro motility assays

In vitro motility assays were performed as described previously [19] with slight modifications. Briefly, 0.2% nitrocellulose-coated coverslips

were coated with *Physarum* myosin by standing on ice for 10 min in a buffer consisting of 60 mM KCl, 25 mM imidazole (pH 7.5), 4 mM $MgCl_2$, and 1 mM DTT; actin filaments (3 nM) labeled with rhodamine–phalloidin (Molecular Probes, Eugene, USA) were introduced into flow cells that were constructed between a glass slide and a coverslip coated with *Physarum* myosin in motility buffer containing 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 various concentrations of Ca^{2+} (pCa: 8, 7, 6, 5, and 4), along with anti-oxidization reagents consisting of 0.2 mg/ml glucose oxidase, 0.04 mg/ml catalase, and 4.5 mg/ml glucose. Movements of actin filaments were recorded using a fluorescence microscope equipped with a silicone-intensifier target camera. Average velocities were determined using WCIF ImageJ and were presented as the means ± standard deviations (SD) of at least 60 actin filaments for each experiment.

Other procedures

Protein concentrations were determined using Bio-Rad protein assays [20] with bovine serum albumin as a standard. SDS-PAGE was performed on 12.5% polyacrylamide gels using the Laemmli buffer system [21] with slight modifications. Statistical significance was determined by Student's *t*-test or one-way ANOVA using Sigma Stat version 3.1. A *P*-value <0.05 was considered statistically significant.

Results

Expression and purification of *Physarum* hybrid myosin constructs

Figure 1A shows the illustration of six constructs with a Ph·HC and *Physarum* and/or scallop LCs: wild-type (Ph·HC/PhRLC/PhELC; **Fig. 1A**, model 1); three hybrids (Ph·HC/ScRLC/ScELC, Ph·HC/ScRLC/PhELC, and Ph·HC/PhRLC/ScELC; **Fig. 1A**, models 2, 3, and 4); and two mutants (Ph·HC/PhRLC/PhELC-3A and Ph·HC/ScRLC/PhELC-3A; **Fig. 1A**, models 5 and 6). To produce hybrid myosins, Sf-9 cells were co-infected with genes for HCs of *Physarum* myosin and LCs of *Physarum* and/or scallop myosin as previously described [14]. After 72 h of culture, co-infected cells were harvested and proteins were purified according to our previous reports [12,14]. SDS-PAGE of purified *Physarum* hybrid myosins (**Fig. 1B**) showed three

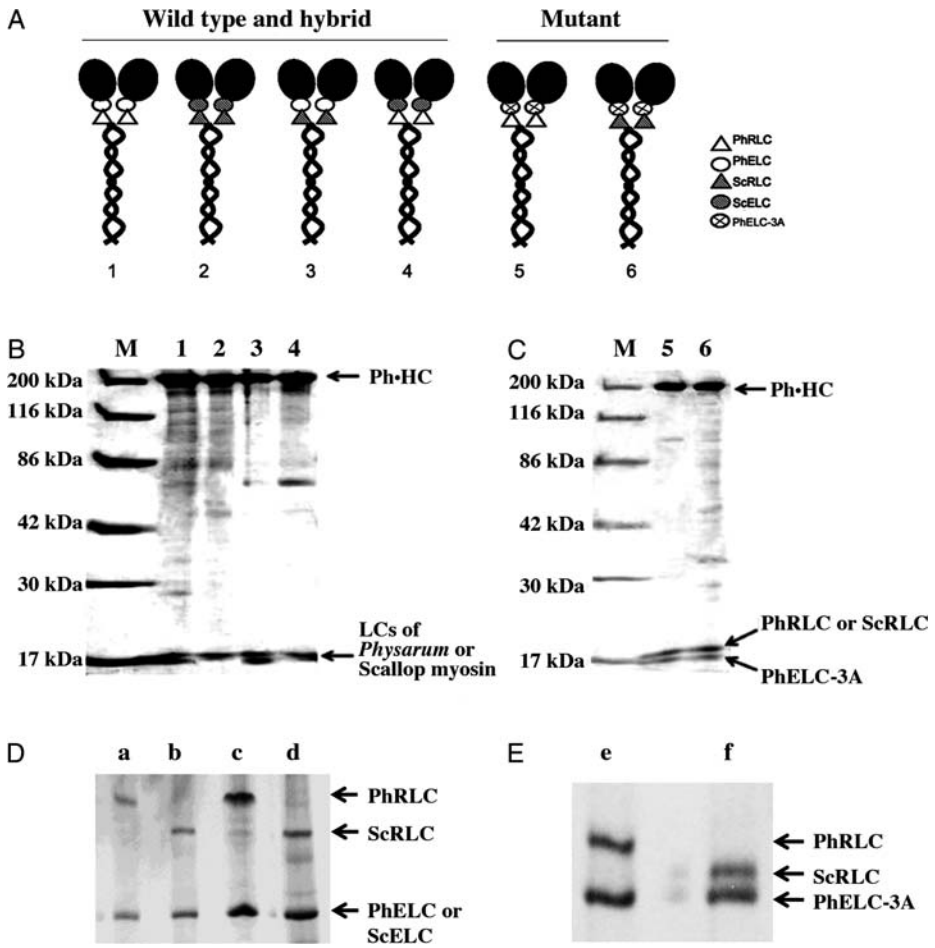

Figure 1. Schematic diagram and purification of *Physarum* myosin hybrid constructs (A) Schematic diagram of *Physarum* myosin hybrid constructs. (B) SDS-PAGE (12.5%) of purified *Physarum* myosin hybrid constructs associated with LCs of *Physarum* and/or scallop myosins. Lane M, molecular weight marker; lane 1, Ph·HC/PhRLC/PhELC; lane 2, Ph·HC/ScRLC/ScELC; lane 3, Ph·HC/ScRLC/PhELC; lane 4, Ph·HC/PhRLC/ScELC. (C) SDS-PAGE (12.5%) of purified myosin mutant Ph·HC/PhRLC/PhELC-3A and hybrid myosin mutant Ph·HC/ScRLC/PhELC-3A. Lane M, molecular weight marker; lane 5, Ph·HC/PhRLC/PhELC-3A; lane 6, Ph·HC/ScRLC/PhELC-3A. (D) Glycerol PAGE showing LCs of expressed *Physarum* hybrid myosin Ph·HC/PhRLC/ScELC (a), expressed *Physarum* hybrid myosin Ph·HC/ScRLC/ScELC (b), *Physarum* myosin (c), and purified scallop myosin (d). (E) Glycerol PAGE showing LCs of expressed *Physarum* myosin mutants of Ph·HC/PhRLC/PhELC-3A (e) and hybrid myosin mutant Ph·HC/ScRLC/PhELC-3A (f).

types of hybrid myosin: (i) Ph·HC/ScRLC/ScELC (lane 2), (ii) Ph·HC/ScRLC/PhELC (lane 3), and (iii) Ph·HC/PhRLC/ScELC (lane 4). Because the molecular weights of ScRLC, ScELC, and PhRLC are close (≈18 kDa), they are indistinguishable by SDS-PAGE; the association of them with the hybrid *Physarum* myosin was confirmed by separating the LCs with glycerol PAGE (**Fig. 1D**). In addition, two *Physarum* myosin mutants were isolated as shown in **Fig. 1**, Ph·HC/PhRLC/PhELC-3A (**Fig. 1C**, lane 5) and Ph·HC/ScRLC/PhELC-3A (**Fig. 1C**, lane 6). As shown in glycerol PAGE (**Fig. 1E**), two LCs of them were clearly visible. All *Physarum* myosin constructs contained HC, ELC, and RLC with a stoichiometry of 1.0:0.9–1.1:0.9–0.97 as determined by using Image J software to analyze the density of protein bands based on the previous reports [22,23].

Effects of Ca^{2+} on ATPase activities of *Physarum* hybrid myosin constructs

To elucidate the role of PhRLC on Ca^{2+}-mediated regulation of myosins, a hybrid myosin Ph·HC/ScRLC/PhELC was constructed by substituting ScRLC for PhRLC and the actin-activated ATPase activities were measured in the presence or absence of Ca^{2+} (**Fig. 2A,B**). Subsequent ATPase activity assays showed reduced activity of the *Physarum* myosin hybrid Ph·HC/ScRLC/PhELC, from 0.592 ± 0.050 s^{-1} in 0.1 mM EGTA to 0.119 ± 0.029 s^{-1} in 0.1 mM Ca^{2+} (pCa: 4), suggesting that PhRLC is not necessary for Ca^{2+}-mediated inhibitory regulation of *Physarum* myosin. Furthermore, a hybrid mutant myosin Ph·HC/ScRLC/PhELC-3A was constructed by substituting PhELC-3A for PhELC, which showed actin-activated ATPase activities of 0.723 ± 0.039 s^{-1} in 0.1 mM EGTA and 0.705 ± 0.029 s^{-1} in 0.1 mM Ca^{2+} (pCa: 4). These data showed no Ca^{2+} inhibition of ATPase activity of this *Physarum* hybrid mutant myosin. To compare the effects of Ca^{2+}, the measurement of ATPase activity was repeated on both *Physarum* myosin wild-type Ph·HC/PhRLC/PhELC and mutant Ph·HC/PhRLC/PhELC-3A. The ATPase activity of Ph·HC/PhRLC/PhELC was clearly inhibited by Ca^{2+} from 1.382 ± 0.142 s^{-1} in 0.1 mM EGTA to 0.046 ± 0.016 s^{-1} in 0.1 mM Ca^{2+} (pCa: 4). However, Ca^{2+} did not inhibit ATPase activity of the *Physarum* myosin mutant Ph·HC/PhRLC/PhELC-3A, as indicated in our previous report [12].

In further experiments, we substituted ScELC for PhELC to obtain another hybrid myosin construct Ph·HC/PhRLC/ScELC. The ATPase activity of this *Physarum* hybrid myosin was reduced from 0.314 ± 0.016 s^{-1} in 0.1 mM EGTA to 0.189 ± 0.008 s^{-1} in 0.1 mM Ca^{2+} (pCa, 4). In addition, after substituting two LCs from scallop myosin (ScRLC and ScELC) for PhRLC and PhELC, the ATPase activity of the consequent hybrid myosin construct was 0.115 ± 0.011 s^{-1} in 0.1 mM EGTA and 0.117 ± 0.016 s^{-1} in 0.1 mM Ca^{2+}, indicating no inhibition by Ca^{2+} (pCa: 4) (**Fig. 2A,B**).

Effects of Ca^{2+} on motor activity of *Physarum* hybrid myosin constructs

Ca^{2+}-dependent regulation of motor activities of expressed *Physarum* myosin constructs was determined using *in vitro* motility assays. In these experiments, glass coverslip surfaces were coated with six different *Physarum* myosin constructs, and movements of actin filaments were monitored (**Supplementary Fig. S1**). Initially, the velocity of actin filaments in the presence of *Physarum* myosin hybrid Ph·HC/ScRLC/PhELC in the presence of 0.1 mM EGTA was 0.389 ± 0.087 μm/s (**Fig. 3B** and **Supplementary Video S1**). However, in the presence of 0.1 mM Ca^{2+} (pCa: 4), no movement was observed (**Fig. 3B** and **Supplementary Video S2**). In subsequent experiments with the *Physarum* myosin hybrid mutant Ph·HC/ScRLC/PhELC-3A, actin filaments moved at a velocity of 0.412 ± 0.087 μm/s in the presence of 0.1 mM EGTA and 0.389 ± 0.020 μm/s in the presence of 0.1 mM Ca^{2+} (pCa: 4) (**Fig. 3D**). In addition, to confirm the accuracy of these results, we repeated the previous published experiments with the constructs of Ph·HC/PhRLC/PhELC and Ph·HC/PhRLC/PhELC-3A [12]. Ca^{2+} remarkably inhibited the movements of actin filaments in the presence of the *Physarum* myosin Ph·HC/PhRLC/PhELC (**Fig. 3A**) but did not affect that of the *Physarum* myosin mutant Ph·HC/PhRLC/PhELC-3A (**Fig. 3C**). These observations were consistent with our previous report that PhELC played an important role on Ca^{2+}-mediated regulation [12]. However, no movements of actin filaments were observed on glass coated with *Physarum* hybrid myosins Ph·HC/PhRLC/ScELC or Ph·HC/ScRLC/ScELC (**Fig. 3E**).

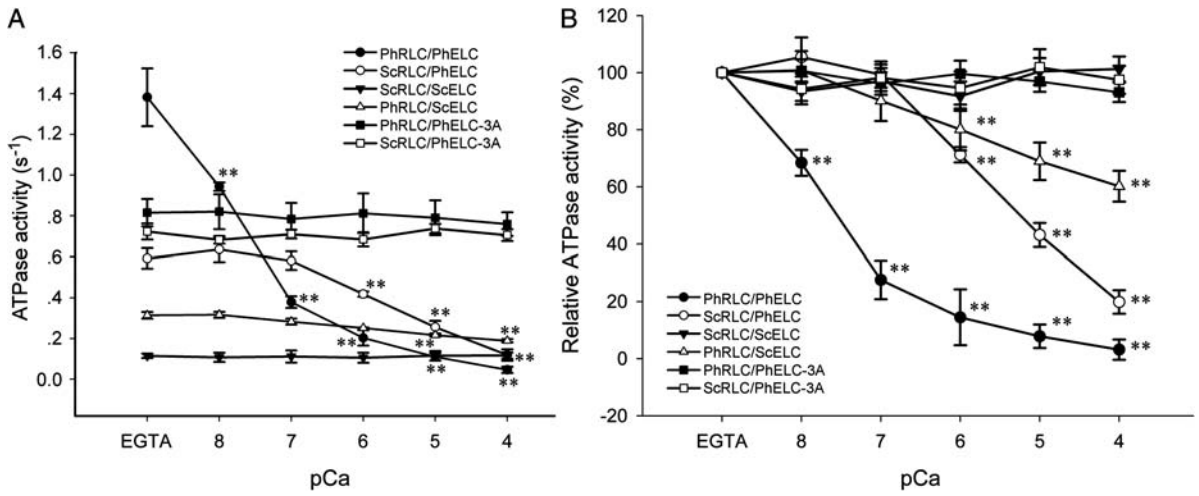

Figure 2. Specific actin-activated ATPase activities of *Physarum* myosin hybrid constructs in EGTA and in the presence of various concentrations of Ca^{2+}
(A) ATPase activity was measured in the presence of *Physarum* myosin hybrid constructs in EGTA and at different concentrations of Ca^{2+} as described in 'Materials and Methods' section. (B) Relative effects of Ca^{2+} on *Physarum* myosin hybrid constructs. Data are presented as means ± SEM, $n = 5$. $^{**}P < 0.01$.

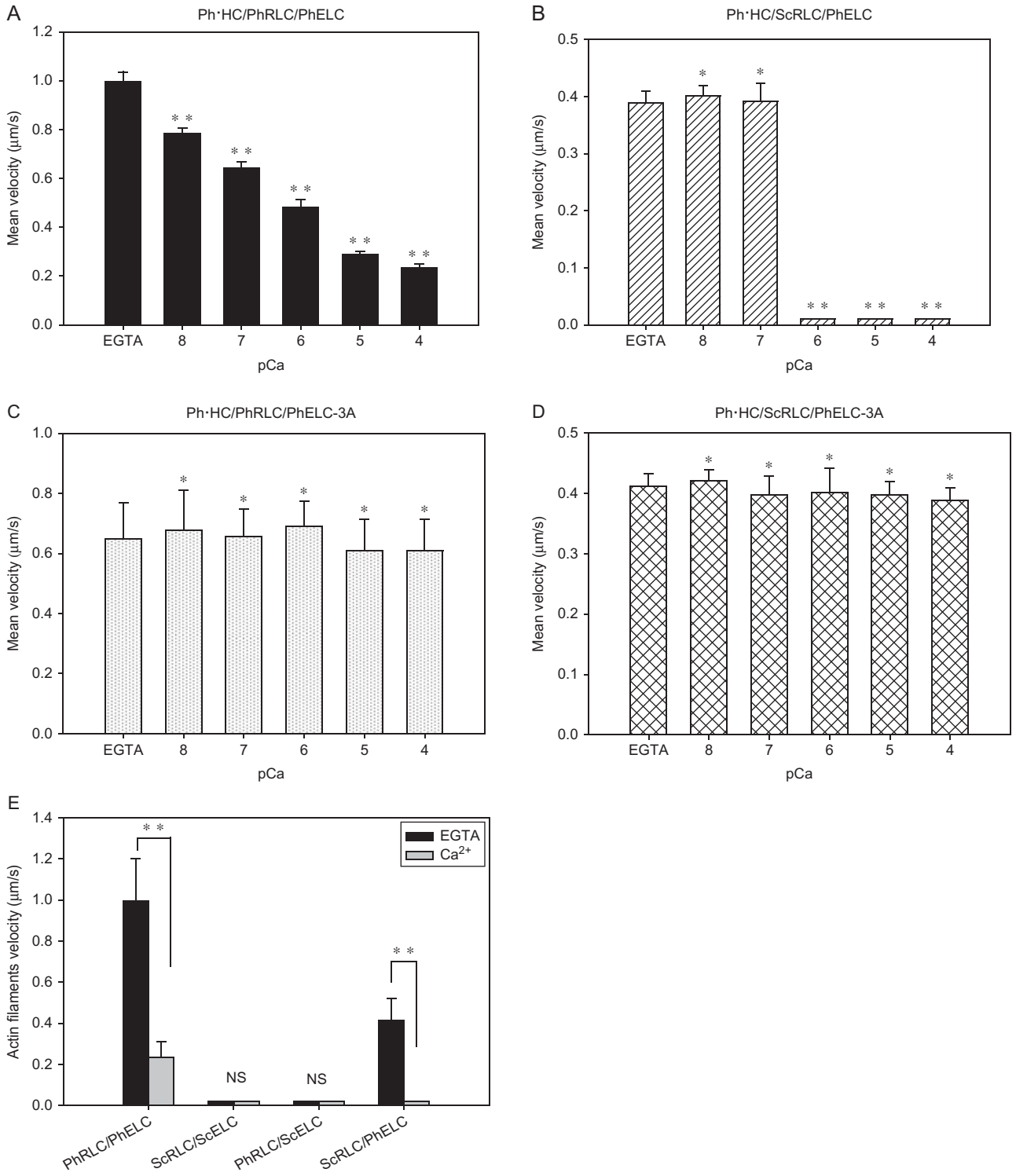

Figure 3. Effects of Ca^{2+} on the velocity of actin filaments on *Physarum* myosin hybrid constructs (A) Velocity of actin filaments on *Physarum* myosin Ph·HC/PhRLC/PhELC in EGTA or various concentrations of Ca^{2+}. (B) Velocity of actin filaments on *Physarum* hybrid myosin Ph·HC/ScRLC/PhELC in the presence of EGTA or various concentrations of Ca^{2+}. (C) Velocity of actin filaments on the *Physarum* myosin mutant Ph·HC/PhRLC/PhELC-3A in the presence of EGTA or various concentrations of Ca^{2+}. (D) Velocity of actin filaments on the *Physarum* hybrid myosin mutant Ph·HC/ScRLC/PhELC-3A in the presence of EGTA or various concentrations of Ca^{2+}. (E) Effects of Ca^{2+} on the velocity of actin filaments on *Physarum* myosin constructs with LCs from *Physarum* (PhRLC and PhELC) and scallop myosin (ScRLC and ScELC) in the presence of EGTA or Ca^{2+}. Data are presented as means ± SD, $n \geq 60$. $^{**}P < 0.01$; $^{*}P > 0.05$; NS, no significant.

Discussion

In the present study, to elucidate the roles of LCs from different species (*Physarum* and scallop) in Ca^{2+}-mediated regulation, we generated four recombinant, full-length *Physarum* myosin constructs (Ph·HC/PhRLC/PhELC, Ph·HC/ScRLC/ScELC, Ph·HC/ScRLC/PhELC, and Ph·HC/PhRLC/ScELC) comprising Ph·HC associated with *Physarum* and/or scallop LCs in Sf-9 cells. In addition, we produced two *Physarum* mutant myosins (Ph·HC/PhRLC/PhELC-3A and Ph·HC/ScRLC/PhELC-3A) comprising Ph·HC associated with ScRLC and PhELC-3A. Subsequent examinations of Ca^{2+}-dependent regulation of motor and ATPase activities indicate that PhELC plays a critical role in Ca^{2+}-mediated regulation of *Physarum* myosin. Although PhRLC is not necessary for Ca^{2+}-mediated regulation of *Physarum* myosin, the presence of PhRLC and PhELC leads to the highest degree of Ca^{2+} regulation of *Physarum* myosins.

In addition, we used Sf-9 cells to express recombinant *Physarum* hybrid myosins. Our preparations were pure, except for a potential contaminant from the native Sf-9 myosin, which may possibly associate with the recombinant myosin during purification. We were unable to estimate the presence and the exact amount of this contaminant, but it is believed to be equal in all preparations, and thus should not significantly affect the comparison between the myosin species.

According to the ATPase assays, the ATPase activities of *Physarum* myosins associated with *Physarum* and/or scallop LCs showed considerable changes in 0.1 mM EGTA (**Fig. 2A**). Their ATPase activities showed the following order: Ph·HC/PhRLC/PhELC > Ph·HC/PhRLC/PhELC-3A > Ph·HC/ScRLC/PhELC-3A > Ph·HC/ScRLC/PhELC > Ph·HC/PhRLC/ScELC > Ph·HC/ScRLC/ScELC, suggesting that the presence of the PhELC is important for maintaining ATPase activity (even at low calcium). In addition, wild-type myosin showed higher activity than any other hybrid myosins. This could be due to that the hybrid myosins are not functioning as optimal as the wild-type due to the structural differences noted in the next discussion. On the other hand, Ph·HC/PhRLC/PhELC exhibited remarkable activity changes in 0.1 mM Ca^{2+} relative to those in 0.1 mM EGTA (96.67% inhibitory effect; **Table 1**). However, Ph·HC/ScRLC/ScELC was almost impervious to Ca^{2+} concentrations (**Fig. 2** and **Table 1**), suggesting that PhRLC and PhELC, but not Ph·HC, mediate Ca^{2+} inhibitory regulation of *Physarum* myosin. However, whereas Ph·HC/PhRLC/ScELC showed a 39.81% inhibitory effect of 0.1 mM Ca^{2+}, greater inhibitory effects were observed with Ph·HC/ScRLC/PhELC in 0.1 mM Ca^{2+} (79.90% inhibitory effect; **Table 1**), indicating that PhELC plays a more important role in Ca^{2+} inhibition than PhRLC. These observations were also confirmed in assays of actin filament motility and ATPase activities of *Physarum* myosin mutant Ph·HC/PhRLC/PhELC-3A [12] and Ph·HC/ScRLC/PhELC-3A, which showed no inhibitory effect of Ca^{2+} (**Figs. 2** and **3C,D**, and **Table 1**). Moreover, the effects of Ca^{2+} on the ATPase activity of the *Physarum* myosin hybrid Ph·HC/ScRLC/PhELC and Ph·HC/PhRLC/ScELC (79.90% and 39.81% inhibitory effects; **Table 1**) were less obvious than that observed with the *Physarum* myosin Ph·HC/PhRLC/PhELC (96.67% inhibitory effect). These results suggest that the presence of PhRLC and PhELC facilitates the full inhibitory effect of Ca^{2+}.

Although we were not able to determine the velocity of movements of actin filaments with *Physarum* myosin hybrid Ph·HC/PhRLC/ScELC or Ph·HC/ScRLC/ScELC, the waggling movements of actin filaments in EGTA were observed using the fluorescence microscope (**Supplementary Videos S3** and **S4**). These observations suggest that interactions between actin and myosin were intact and were consistent with a previous report [16]. Moreover, lower ATPase activities of *Physarum* myosin hybrids Ph·HC/PhRLC/ScELC and Ph·HC/ScRLC/ScELC than that of *Physarum* myosin wild-type were observed in EGTA (**Fig. 2**), indicating that they may not be able to slide the actin filament. VanBuren *et al.* [24] showed that ELC is required for full force production by skeletal muscle myosin, and ELC removal reduced both force and velocity more than RLC removal in skeletal muscle myosin. In addition, the ELC is closer to the motor domain and thus the replacement of the ELC is more disruptive than replacing or removing the RLC [25,26]. It is therefore not too surprising that no movements of actin filaments on the *Physarum* myosin hybrids Ph·HC/PhRLC/ScELC and Ph·HC/ScRLC/ScELC were observed.

Table 1. The relative actin-activated ATPase activities of *Physarum* myosin constructs in EGTA and in Ca^{2+} and the effects of Ca^{2+} on them

Physarum myosin constructs	The relative actin-activated ATPase activities		
	EGTA (%)[a]	Ca^{2+} (%)[b]	Effect of Ca^{2+} (%)[c]
Ph·HC/PhRLC/PhELC	100	3.33	96.6↓↓↓
Ph·HC/ScRLC/PhELC	100	20.10	79.90↓↓
Ph·HC/PhRLC/ScELC	100	60.19	39.81 ↓
Ph·HC/ScRLC/ScELC	100	101.74	1.74↑
Ph·HC/PhRLC/PhELC-3A	100	93.13	6.87↓
Ph·HC/ScRLC/PhELC-3A	100	97.51	2.49↓

[a]The actin-activated ATPase activities of *Physarum* myosin constructs were considered as 100% in EGTA.

[b]The actin-activated ATPase activities in Ca^{2+} / in EGTA × 100.

[c]Effect of Ca^{2+} (%)[c] = |Ca^{2+} (%)[b] − EGTA (%)[a]|/EGTA (%)[a] × 100; ↓means inhibition; ↑ means little activation and no significant difference; ↓means little inhibition and no significant difference.

Physarum polycephalum, a lower eukaryote, shows vigorous shuttling of cytoplasmic streaming [27]. This cytoplasmic streaming is regulated by Ca^{2+} and driven by a conventional myosin II; the increase in [Ca^{2+}]i is a signal for myosin activation and the decrease in [Ca^{2+}]i for myosin inhibition [10]. This regulatory mode was confirmed in the living plasmodium in our recent study and the Ca^{2+} regulation was observed in recombinant myosin purified from Sf-9 cells [12,28]. However, it is unclear how this regulation plays a role in its physiological function. Similar to *Physarum* myosin, regulation of scallop myosin is also controlled by direct binding of Ca^{2+} to the ELC [7]. However, the effect of Ca^{2+} is totally opposite to that of *Physarum* myosin; Ca^{2+} activates its activity [7,11]. Further biochemical studies demonstrated that Ca^{2+} binding to the ELC to inhibit/activate the ATPase activity and *in vitro* motility [13]. Structural studies showed that the Ca^{2+} inhibition/activation difference between the two conventional myosins may be due to difference in Ca^{2+}-induced change in the conformational flexibility of the regulatory domain (RD) comprising ELC, RLC, and a portion of the HC [25]. By comparing the amino acid sequence of PhELC with that of ScELC, we found relatively low sequence similarity between these two Ca^{2+}-binding ELCs (**Fig. 4A**). In addition, methionine is absent in the N-lobe of PhELC and only one methionine exists in ScELC (**Fig. 4A**). Interestingly, the larger numbers of methionines in CaM (more than 5%) are thought to adopt the open

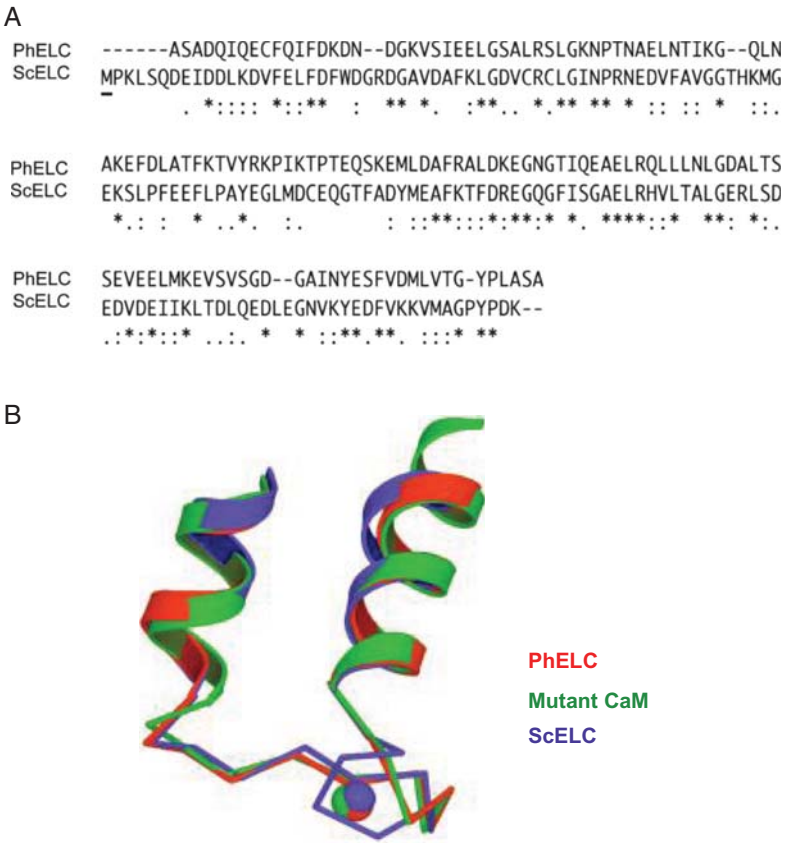

Figure 4. Comparisons of amino acid sequences and structure of the Ca^{2+}-binding EF-hands between PhELC and ScELC (A) Comparisons of amino acid sequences between PhELC and ScELC using the program of CLUSTAL W (1.83). The first row shows amino acid sequences of PhELC and the second row shows that of ScELC. '*' indicates positions that have a single, fully conserved residue; ':' indicates that 'strong' group is fully conserved; '.' indicates that 'weaker' group is fully conserved. The underlined letter 'M' in the beginning of second row indicates the only one methionine in N-lobe of ScELC. (B) Structure and comparison of the Ca^{2+}-binding EF-hand I of PhELC (red), ScELC (blue), and mutant CaM (green) [25]. Note that the extra turn in the first helix of ScELC. Adapted from Debreczeni *et al.* [25].

conformation in Ca^{2+}-binding state [29]. Accordingly, the absence of methionine in the PhELC may prevent the large conformational transition. By comparison of the structure of Ca^{2+}-binding EF-hands of PhELC with that of ScELC, an extra turn in the ScELC was observed (**Fig. 4B**). These differences between PhELC and ScELC may result in conformational flexibility or intramolecular interactions between Ph·HC and ScELC that are not appropriate.

Based on these results, we have proposed a simple model to explain the regulatory effect of Ca^{2+}. In EGTA condition (in Ca^{2+}-free state), *Physarum* myosin is the active 'open' state in which the myosin shows high actin-activated ATPase activity and induces the sliding movement of actin. This 'open' state of *Physarum* myosin may require that the PhRLC and the PhELC maintain specific orientations relative to each other so that active conformation can be formed. Substitution of the PhRLC or the PhELC could not, however, produce a normal 'open' state. Ca^{2+}-binding to the PhELC may alter the flexibility of the RD and induce a 'closed' state, which may consequently prevent full activity and force generation. Although the opposite effects of Ca^{2+} are seen on *Physarum* and scallop myosin, the effects of Ca^{2+}-binding to the ELC on the changes of RD flexibility seem to be similar [25,30]. To fully understand the opposite regulatory effect of Ca^{2+}-binding on *Physarum* and scallop myosin clearly, further studies should be carried out to express scallop myosin HC with the *Physarum* LCs and determine if the LCs can reverse the regulation of Ca^{2+}.

Supplementary Data

Supplementary data is available at *ABBS* online.

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References

1. Sellers JR. Regulation of cytoplasmic and smooth muscle myosin. *Curr Opin Cell Biol* 1991, 3: 98–104.
2. Matsumura F, Hartshorne DJ. Myosin phosphatase target subunit: many roles in cell function. *Biochem Biophys Res Commun* 2008, 369: 149–156.
3. Hartman MA, Spudich JA. The myosin superfamily at a glance. *J Cell Sci* 2012, 125: 1627–1632.

4. Zhang Y, Zhang H, Tang Z, Kohama K, Lin Y. Inverse interaction between tropomyosin and phosphorylated myosin in the presence or absence of caldesmon. *Acta Biochim Biophys Sin (Shanghai)* 2013, 45: 601–606.
5. Yang JX, Feng XH, Zhang Y, Lin Y. Influence of trace amount of calponin on smooth muscle myosin in different states. *Acta Biochim Biophys Sin (Shanghai)* 2004, 36: 529–536.
6. Allen BG, Walsh MP. The biochemical basis of the regulation of smooth-muscle contraction. *Trends Biochem Sci* 1994, 19: 362–368.
7. Szent-Gyorgyi AG, Kalabokis VN, Perreault-Micale CL. Regulation by molluscan myosins. *Mol Cell Biochem* 1999, 190: 55–62.
8. Chantler PD. Scallop adductor muscles: structure and function. In: Parsons GJ, Shumway SE eds. *Scallops: Biology, Ecology and Aquaculture*. 2nd edn. Amsterdam: Elsevier, 2005, 231–289.
9. Kawamichi H, Zhang Y, Hino M, Nakamura A, Tanaka H, Farkas L, Nyitray L, *et al.* Calcium inhibition of *Physarum* myosin as examined by the recombinant heavy mero-myosin. *Adv Exp Med Biol* 2007, 592: 265–272.
10. Kohama K. Ca-inhibitory myosins: their structure and function. *Adv Biophys* 1987, 23: 149–182.
11. Kalabokis VN, Szent-Györgyi AG. Regulation of scallop myosin by calcium: cooperativity and the "off" state. *Adv Exp Med Biol* 1998, 453: 235–240.
12. Zhang Y, Kawamichi H, Tanaka H, Yoshiyama S, Kohama K, Nakamura A. Calcium-dependent regulation of the motor activity of recombinant full-length *Physarum* myosin. *J Biochem* 2012, 152: 185–190.
13. Okagaki T, Higashi-Fujime S, Kohama K. Ca^{2+} activates actin-filament sliding on scallop myosin but inhibits that on *Physarum* myosin. *J Biochem* 1989, 106: 955–957.
14. Zhang Y, Nakamura A, Kawamichi H, Yoshiyama S, Katayama T, Kohama K. Calcium regulation of the ATPase activity of *Physarum* and scallop myosins using hybrid smooth muscle myosin: the role of the essential light chain. *FEBS Lett* 2010, 584: 3486–3491.
15. Himmel DM, Mui S, O'Neall-Hennessey E, Szent-Györgyi AG, Cohen C. The on-off switch in regulated myosins: different triggers but related mechanisms. *J Mol Biol* 2009, 394: 496–505.
16. Al-Khayat HA, Morris EP, Squire JM. The 7-stranded structure of relaxed scallop muscle myosin filaments: support for a common head configuration in myosin-regulated muscles. *J Struct Biol* 2009, 166: 183–194.
17. Spudich JA, Watt S. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J Biol Chem* 1971, 246: 4866–4871.
18. Webb MR. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc Natl Acad Sci USA* 1992, 89: 4884–4887.
19. Ishikawa R, Okagaki T, Higashi-Fujime S, Kohama K. Stimulation of the interaction between actin and myosin by *Physarum* caldesmon-like protein and smooth muscle caldesmon. *J Biol Chem* 1991, 266: 21784–21790.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, 72: 248–254.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227: 680–685.
22. Gassmann M, Grenacher B, Rohde B, Vogel J. Quantifying western blots: pitfalls of densitometry. *Electrophoresis* 2009, 30: 1845–1855.
23. Tan HY, Ng TW. Accurate step wedge calibration for densitometry of electrophoresis gels. *Opt Commun* 2008, 281: 3013–3017.
24. VanBuren P, Waller GS, Harris DE, Trybus KM, Warshaw DM, Lowey S. The essential light chain is required for full force production by skeletal muscle myosin. *Proc Natl Acad Sci USA* 1994, 20: 12403–12407.
25. Debreczeni JE, Farkas L, Harmat V, Hetenyi C, Hajdu I, Zavodszky P, Kohama K, *et al.* Structural evidence for non-canonical binding of Ca^{2+} to a canonical EF-hand of a conventional myosin. *J Biol Chem* 2005, 280: 41458–41464.
26. Rayment I, Rypniewski WR, Schmidt-Bäse K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, *et al.* Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science* 1993, 261: 50–58.
27. Yoshimoto Y, Matsumura F, Kamiya N. Simultaneous oscillations of Ca^{2+} efflux and tension generation in the permealized plasmodial strand of *Physarum*. *Cell Motil* 1981, 1: 433–443.
28. Yoshiyama S, Ishigami M, Nakamura A, Kohama K. Calcium wave for cytoplasmic streaming of *Physarum polycephalum*. *Cell Biol Int* 2009, 34: 35–40.
29. Nelson MR, Chazin WJ. An interaction-based analysis of calcium-induced conformational changes in Ca^{2+} sensor proteins. *Protein Sci* 1998, 7: 270–282.
30. Gourinath S, Himmel DM, Brown JH, Reshetnikova L, Szent-Gyorgyi AG, Cohen C. Crystal structure of scallop myosin S1 in the pre-power stroke state to 2.6 a resolution: flexibility and function in the head. *Structure* 2003, 11: 1621–1627.