Review

Calcium inhibition as an intracellular signal for actin–myosin interaction

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Abstract: Intracellular signaling pathways include both the activation and the inhibition of biological processes. The activation of Ca$^{2+}$ regulation of actin-myosin interactions was examined first, whereas it took 20 years for the author to clarify the inhibitory mode by using Physarum polycephalum, a lower eukaryote. This review describes the investigation of the inhibitory mode since 1980. The inhibitory effect of Ca$^{2+}$ on myosin was detected chemically by ATPase assays and mechanically by in vitro motility assays. The Ca$^{2+}$-binding ability of Physarum myosin is as high as that of scallop myosin. Ca$^{2+}$ inhibits Physarum myosin, whereas it activates scallop myosin. We cloned cDNA of the myosin heavy chain and light chains to express a hybrid of Physarum and scallop myosin, and found that the Ca-binding light chain (CaLc), which belongs to an alkali light chain class, plays a major role in Ca inhibition. The role of CaLc was confirmed by mutating its EF-hand, Ca-binding structure and expressing Physarum myosin as a recombinant protein. Thus, the data obtained by classical protein purification were confirmed by the results obtained with the modern recombinant techniques. However, there are some discrepancies that remain to be solved as described in Section XII.

Keywords: Physarum polycephalum, actin-myosin interaction, calcium regulation, inhibitory effect of Ca$^{2+}$, phosphorylation, recombinant protein

I. Introduction

The latter half of 1970s, when I started working with Physarum polycephalum, was an exciting era. The field of actomyosin expanded from muscle cells to non-muscle cells, including a variety of vertebrate and invertebrate cells. In muscle cells, actomyosin is a system responsible solely for contraction. However, in non-muscle cells, it is a major component of the cytoskeleton, and is responsible for various physiological functions. Our understanding of the regulatory role of Ca$^{2+}$ at the micromolar level was also developing owing to the discovery of Ca-binding proteins, such as troponin and calmodulin expanded our knowledge of the regulatory role of Ca$^{2+}$ beyond actomyosin regulation.$^{1}$

I was educated as a protein biochemist of muscle tissues, and was interested in Physarum, a lower eukaryote that shows vigorous shuttle streaming in the cytoplasm, the force of which is generated by actomyosin. Physarum was a good model organism for protein chemistry, because it can be cultured in the lab in large quantities,$^{2}$ and because the procedures for purifying actin$^3$ and myosin$^4$ were similar to those for skeletal muscle. It was expected that Ca$^{2+}$ would regulate actin and myosin in Physarum in a similar way to muscle; namely, that Ca$^{2+}$ would activate the actin-myosin interaction. However, this review describes that the regulation is quite different in terms of Ca$^{2+}$ regulation.

At first, I purified actomyosin preparations, which were thought to be composed of actin, myosin, and regulatory proteins. The effect of Ca$^{2+}$ was examined by inducing ATP-dependent aggregation
of actin and myosin under a spectrophotometer. When the aggregation as named by superprecipitation was induced in EGTA, i.e., in the absence of Ca\(^{2+}\), it was more rapid than in the presence of Ca\(^{2+}\). The effect was the opposite of the known effect of Ca\(^{2+}\), which activated the ATP-dependent interaction between actin and myosin. The effect was not reproducible when I first observed the inhibitory effect of Ca\(^{2+}\). However, Ca-inhibitory actomyosin was prepared consistently by repeated washing of the actomyosin preparation, by dissolution in a high-salt buffer followed by precipitation by dilution of the solution with cold water. The result was published in the Proceedings of the Japan Academy Series B in a communication by my supervisor, Professor Setsuro Ebashi.\(^5\)

This review describes how the inhibitory effect of Ca\(^{2+}\) on Physarum actomyosin has been discovered. As shown in Table 1, the cytoplasmic concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]i) is kept as low as possible by the extrusion of Ca\(^{2+}\) through the cell membrane and by the sequestration of Ca\(^{2+}\) in the cytoplasmic reticulum. When the cell is excited, [Ca\(^{2+}\)]i increases owing to the entry of extracellular Ca\(^{2+}\) and the release of sequestered Ca\(^{2+}\). The increase soon disappears, because the mechanisms for keeping [Ca\(^{2+}\)]i low are triggered again. Cells use this transient increase in [Ca\(^{2+}\)]i as a secondary messenger to regulate the actin–myosin interaction,\(^6\) both in the animal and plant kingdoms. However, the modes of use are different; intracellular Ca\(^{2+}\) is an inhibitor for plant cells and an activator for animal cells. One of the conclusions of this review is that the plant mode of use is observed in Physarum.

I wrote this review as a personal memoir of over 40 years’ research on Physarum. It is based on our published reviews\(^2\),7–22\) and on recent original publications.\(^23\),24\) I would like to describe how Ca\(^{2+}\) exerts a regulatory effect on actomyosin via the inhibitory mode of Ca regulation.

### II. Purification of Physarum myosin

A method for preparing Ca-inhibitory myosin in its actin–myosin interaction was developed by Kohama and Kendrick-Jones.\(^25\) It was based on the preparation of actomyosin, from which myosin is rapidly purified (Table 2).

The procedure for preparing actomyosin is a modification of the method published by Hatano and Tazawa.\(^26\) Actomyosin is extracted by homogenizing Physarum plasmodial cells in a high-salt buffer (pH 7.8–8.0) containing EGTA, followed by the removal of cell debris and slime by centrifugation at 50,000 \(\times\) g for 30 min (Steps 1 and 2). Then, the crude actomyosin is recovered from the centrifugation supernatant as a precipitate produced by reducing the pH of the buffer to 6.5 and the ionic strength to about 50 mM. Native actomyosin is purified from the crude actomyosin preparation by

| Table 2. Purification of calcium inhibitory myosin |
|----------------------------------|
| **Step 1** Culture plasmodium of Physarum polycephalum in the dark on rolled oats. |
| **Step 2** Extract in high salt buffer after homogenization of the plasmodium. |
| **Step 3** · Precipitate actomyosin by diluting the extract, followed by dissolving by the high salt buffer.  
  · Repeat the dissolution-precipitation cycles, giving calcium inhibitory actomyosin. |
| **Step 4** · Solubilize, the actomyosin preparation in the solution containing 20 mM ATP.  
  · Add concentrated Mg acetate stock to give 0.1 M to assemble actin-filaments into bundles.  
  · Remove bundles by the centrifugation. |
| **Step 5** · Collect the supernatant of which myosin was almost actin free.  
  · Dilute the supernate by adding 2 vol of cold water to precipitate myosin by forming myosin filaments.  
  · The precipitate was used as Physarum myosin, showing calcium inhibition. |

Note that column chromatographic procedures are not employed in this preparation, which enables one to prepare Physarum myosin rapidly, i.e., Physarum myosin is recovered as a final purified form within 2 days. For detail, see Ref. 25.

We interpret reactive SH residues as explained in Section II in the myosin heads is protected from the oxidation during the short purification procedures.
repeating cycles of dissolution in high-salt buffer and precipitation in low-salt buffer (Step 3).

Myosin is purified by a modification of the method that Ebashi developed for removing actin from smooth muscle actomyosin preparation.27) The native actomyosin is dissolved in 20 mM ATP containing DTT, mixed with concentrated Mg acetate solution to give a final concentration of 0.1 M, and centrifuged at 100,000 \( \times g \) for 30 min (Step 4). The supernatant is mixed with 2 volumes of cold water, and allowed to stand for 3–4 h on ice; myosin is then recovered as a precipitate (Step 5). Because this myosin preparation was often contaminated with a trace amount of actin, as shown by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), we usually repeated Step 5.

Thus, myosin was purified rapidly (within 2 days) to a high level (>95% by SDS-PAGE) and in high yield (about 10 mg/100 g packed wet cells). The ratio of absorbance at 280 nm to that at 260 nm was about 1.5, indicating a low level of nucleotide or RNA contamination.

When Ca-inhibitory myosin was treated with N-ethylmaleimide (NEM), the inhibitory effect of Ca\(^{2+}\) on the myosin disappeared.28) The role of highly reactive SH groups in myosin activity are known in skeletal muscle.29) We proposed that the modification by NEM made Physarum myosin insensitive to Ca\(^{2+}\), and that rapid preparative procedures that did not involve column chromatography and long centrifugation might contribute to obtaining intact native myosin.

### III. Subunit composition

As shown in Table 3,7(8,10–13,30,31) myosin is resolved into three subunits by SDS-PAGE: a heavy chain (HC) with a molecular weight of 230 kDa, which is larger than the HC of skeletal muscle myosin; and two light chains (Lcs) with molecular weights of 16 and 18 kDa. Quantitative densitometry

| Subunit Composition | Physarum | Skeletal muscle |
|---------------------|-----------|-----------------|
| HC                  | 230 Kd\(^e\) | 200 Kd\(^e\) |
| PLc                 | 18 Kd\(^f\) | 19 Kd\(^f\) |
| CaLc                | 16 Kd (14 Kd\(^f\)) | Al–Lc |
|                     |            | Al\(_1\) = 25 Kd (21 Kd\(^f\)) |
|                     |            | Al\(_2\) = 17 Kd |
| Tail length         | 170 nm     | 150 nm          |
| Rod (M\(_t\))       | >80%       | <20%            |
| Viscosity\(^d\)     | 2.0 dl/g\(^d\) | 1.5 dl/g\(^d\) |
| Assembly (see, Section IV) | 150 mM KCl | No |
|                     | 30 mM KCl  | Yes\(^e\) |
|                     | Bipolar filaments | Short |
|                     | S\(_{20,w}\) | 7.0 S\(^f\) |
| Ca-binding capacity (see, Fig. 6) | 2 mol/mol\(^5\) | 0 mol/mol\(^5\) |

### Myosin ATPase activities

- Ca-ATPase > K-EDTA ATPase > Mg-ATPase
- K-EDTA-ATPase > Ca-ATPase > Mg-ATPase

### Actin-activated Mg-ATPase activities

- Inhibited by \(\mu\)M levels of Ca\(^{2+}\)
- Not affected by Ca\(^{2+}\)

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\(^a\)Physarum plasmodial cell (adopted from Ref. 10). \(^b\)Fast white muscle (adopted from Ref. 31). \(^c\)Estimated by SDS–PAGE. \(^d\)Compared under the same conditions (0.5 M KCl, 3.5 mM Mg acetate, and 20 mM Tris–HCl pH 7.5). \(^e\)Assembly is inhibited by the dephosphorylation. \(^f\)In 0.3 M KCl, 3.5 mM Mg acetate and 20 mM Tris–HCl pH 7.5. \(^g\)Expressed per mol of 2-headed myosin molecule.
of SDS-PAGE shows that the three subunits are present in a 1:1:1 stoichiometry. Myosin is eluted from a gel-filtration column under high salt conditions in the fraction where skeletal muscle myosin is eluted; thus, the myosin molecule is made up of six polypeptides, two of each subunit.

The 18 kDa, phosphorylatable light chain (PLc) bound to skeletal muscle myosin that had 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)-Lc removed by DTNB-treatment. The 16 kDa protein, a Ca-binding light chain (CaLc), did not bind to the DTNB-treated myosin. However, CaLc was exchanged with the alkali Lc (Al1, Al2) of skeletal muscle by KSCN treatment followed by LiCl treatment. Thus, the CaLc of Physarum myosin is similar to the alkali Lc of skeletal muscle myosin, and the PLc of Physarum myosin is similar to the DTNB-Lc of skeletal muscle myosin.

IV. Head and tail structure

Figure 1D shows a myosin molecule visualized by electron microscopy after rotary shadow casting. The molecule consists of a long rod-shaped tail domain and two globular head domains. The HCs span the entire length of the molecule, whereas the Lcs are associated only with the head region (see Section X). The tail portion is involved in self-assembly; the head portion exhibits ATPase activity, and interacts with actin.

The properties related to the head and tail structures are similar to those of skeletal muscle myosin. Table 3 summarizes the differences in the head and tail structures between Physarum and skeletal muscle myosin. The main differences are as follows: (i) Physarum myosin has a longer tail (170 nm) than skeletal muscle myosin (150 nm), which is consistent with observations that it is more viscous (2.0 dL/g) than skeletal muscle myosin (1.5 dL/g). (ii) The Physarum myosin tail contains one or two sharp (<90°) bends, which are not usually observed in skeletal muscle myosin. (iii) Physarum myosin does not assemble in 150 mM KCl, a physiological salt concentration for skeletal muscle cells, but does assemble to form filaments in 30 mM KCl, a physiological salt concentration for Physarum plasmoidal cells (see Section XI C). The extent of the Physarum myosin assembly is modified by its phosphorylated state. In contrast, skeletal muscle myosin assemblies in 30 and 150 mM KCl irrespective of its phosphorylated state. (iv) Physarum myosin can be obtained as a recombinant protein. As discussed in Section X, these differences will be confirmed by using recombinant Physarum myosin.

V. Physarum myosin ATPase activities

A. Myosin ATPase activities in the absence of actin. Mg-ATPase activity in low-salt buffer containing EGTA (Ca²⁺ < 1 nM) is 4–10 nmol min⁻¹ mg⁻¹ (Table 3), whereas Ca-ATPase activity is typically 70 nmol min⁻¹ mg⁻¹. When assayed at high salt concentrations (0.5 M KCl), Mg-ATPase and Ca-ATPase activities increase to 30 and 800 nmol min⁻¹ mg⁻¹, respectively.

K-EDTA-ATPase activity is typically 100 nmol min⁻¹ mg⁻¹, which is three times that of the Mg-ATPase activity and 1/8 that of Ca-ATPase activity at high salt concentrations. K-EDTA-ATPase activities of myosins from vertebrate muscle and non-muscle cells show the highest myosin ATPase activities. However, Physarum myosin shows the opposite behavior; K-EDTA-ATPase activity is lower than that of Ca-ATPase, which may be a feature of myosin in lower eukaryotes.

B. Myosin Mg-ATPase activity in the presence of actin. A typical experiment examining the effect of actin on myosin Mg-ATPase activity at low salt concentrations is shown in Fig. 1A. The maximum rates of reaction, Vmax, in EGTA and 50 µM Ca²⁺ were 230.0 and 54.1 nmol min⁻¹ mg⁻¹ myosin, respectively. Thus, actin maximally activated Mg-ATPase activity 51.1-fold in the absence of Ca²⁺ and 31.1-fold in the presence of 50 µM Ca²⁺ (see, Section VII). The Michaelis constant, Km, for actin was 0.6 µM, irrespective of the presence or absence of Ca²⁺, suggesting that Ca²⁺ exerted its regulatory effect on myosin after myosin associated with actin. The relationship between Ca²⁺ concentration and actin-activated Mg-ATPase activities (Fig. 1C) indicated that half-maximal inhibition occurred at 1–3 µM Ca²⁺.

C. Inhibitory effect of Ca²⁺: myosin-linked Ca inhibition. Purified actin and myosin from skeletal muscle have no regulatory roles themselves. Mg-ATPase activity of skeletal muscle myosin activated by skeletal muscle actin is not inhibited by Ca²⁺ (Fig. 1C). This Ca-insensitive Mg-ATPase activity of skeletal myosin was also observed when it was activated by Physarum actin. However, in Physarum myosin, the Mg-ATPase activity was inhibited by an increase in Ca²⁺ concentration, irrespective of whether the actin was from Physarum or skeletal muscle. These hybrid experiments clearly demonstrated that the Ca inhibition of actin-
Fig. 1. Characterization of *Physarum* (Ph)-myosin. A. Actomyosin was extracted at high salt concentration as described in Step 1–Step 3 of Table 2, followed by the purification procedures of myosin (Steps 4–5 of Table 2). The extraction of actomyosin was also carried out at low salt concentration, followed by the same myosin purification procedures. The both myosins were subjected to the Mg-ATPase activity measurement. (○), high salt extraction procedure; (□) low salt extraction procedure; (——) in the absence of skeletal muscle (Sk) actin or (-----) in the procedure of Sk actin. Specific activities (nmol·min⁻¹·mg⁻¹ myosin) in the presence of 0.1 mM EGTA (100%) of (●), (○), (△), and (□) were 173.4, 144.4, 4.4, and 9.4, respectively. As described in this Section V, the myosin properties were not altered due to the extraction methods, myosin was prepared mostly by the high salt extraction unless otherwise mentioned. Note that the inhibitory effect of Ca²⁺, i.e., calcium inhibition was obvious only when actin was present, i.e., only when it was measured in the Sk actin-activated Mg-ATPase activity of myosin. B. Calcium binding of Ph-myosin was related to calcium inhibition of Sk actin-activated Mg-ATPase activity. Ph-myosin was allowed to bind ⁴⁵CaCl₂. Note that the inverse relationship between the ATPase activity and Ca-binding activity. C. Myosin-linked nature of calcium inhibition. Actins and myosins were purified from both Sk and plasmodia, and subjected to the actin-activated Mg-ATPase measurement of myosin. The combinations of actin and myosin are as follows; Ph myosin in the presence of Ph actin (●), Ph myosin in the presence of Sk actin (○), Sk myosin in the presence of Ph actin (△), Sk myosin in the presence of Sk actin (□). Note that calcium inhibition was observed only when myosins were purified from Ph. D. Electron micrographs of Ph-myosin. Ph-myosin was sprayed on the mica was visualized by rotary shadow casting. The structure of head and tail indicated Ph-myosin belongs to myosin II family.
activated Mg-ATPase activity of *Physarum* myosin was mediated by *Physarum* myosin.28)  

**VI. Ca-binding properties of *Physarum* myosin**  

**A. Inverse relationship of Ca-binding activity with the ATPase activity of *Physarum* myosin.** *Physarum* myosin bound $^{45}$Ca$^{2+}$ with a high affinity at micromolar dissociation constants28) and with a binding capacity of 2 mol Ca$^{2+}$ per mole of myosin. Figure 1B shows that Ca-binding activity increases with Ca$^{2+}$ concentration. However, ATPase activity decreases with the increase of Ca$^{2+}$ concentration at micromolar levels. The inverse relationship between Ca-binding activity and ATPase activity indicates that Ca-binding activity inhibits ATPase activity.  

**B. Ca-binding light chain as a Ca-receptive subunit of *Physarum* myosin.** *Physarum* myosin is composed of a pair of HCs and two pairs of Lcs of 18 and 16 kDa (Fig. 2Aa). The domain structure of the HC showed that the binding sites for ATP, actin, and Lcs are all in the HCs,12) which was confirmed by cloning and expressing HC cDNA (see Section X). The Ca binding site is localized in the 16-kDa CaLc. The primary structure of CaLc was determined by peptide analysis and cDNA cloning,34) and its molecular weight was calculated to be 16,084 Da. The EF-hand structure, which is a consensus sequence for Ca-binding proteins, was identified at one position at the N terminal. The highest homology of CaLc was found in bovine brain calmodulin.34)  

The evidence that CaLc is a Ca-receptive subunit is as follows (Table 4). (i) The mobility of CaLc in SDS-PAGE of myosin was altered in the
presence of Ca$^{2+}$. (ii) The CaLc band in SDS-PAGE of myosin bound to $^{45}$Ca$^{2+}$ and biochemical measurements using $^{45}$Ca$^{2+}$ showed that CaLc bound 0.4 mol Ca$^{2+}$ per mole. This figure is too low to be explained by Ca-binding (1.3 mol Ca$^{2+}$ per mole) of the parent myosin. It was proposed that the Ca-binding activity of CaLc increases when it is incorporated into the myosin molecule, which was later confirmed by expressing CaLc as a recombinant protein (see Section X).

VII. Phosphorylated states and Ca inhibition of Physarum myosin

A. Phosphorylation and dephosphorylation of Physarum myosin. 1. Phosphorylation sites of Physarum myosin. The total phosphate content after afterashing Physarum myosin was 6.8–4.0 mol P$_i$ per mol myosin (500 kDa), indicating that the myosin is phosphorylated at multiple sites (Fig. 2B). The major phosphorylation sites were in the HC, because the myosin from plasmodial cells cultured in the presence of H$_3$[32P]O$_4$ incorporated the radioactive isotope only in the HC (Figs. 2Ba and b). However, a subspot on two-dimensional isoelectric focusing SDS-PAGE of myosin suggested the partial phosphorylation of PLc (Fig. 2Bc). PLc phosphorylation was also consistent with the observation that PLc was phosphorylated by an endogenous kinase contaminating the myosin preparation (Fig. 2Bb). Myosin from vertebrates can be purified in the phosphorylated state, whereas myosins from lower eukaryotes, such as Physarum, Dictyostelium, and Acanthamoeba were purified in the dephosphorylated state.

2. Dephosphorylation and Ca inhibition. Physarum myosin was dephosphorylated with exogenous acid phosphatase, and its actin-activated ATPase activity was compared with that of untreated, phosphorylated myosin. The activity was high in the absence of Ca$^{2+}$ and decreased as Ca$^{2+}$ concentration increased. The activity of dephosphorylated myosin was high in the absence of Ca$^{2+}$ and remained low in the presence of Ca$^{2+}$ irrespective of changes in Ca$^{2+}$ concentration. We examined the Ca-binding activity of myosin following dephosphorylation. Dephosphorylated myosin binding to Ca$^{2+}$ was similar to that of phosphorylated myosin (Table 5). Therefore, dephosphorylation of Physarum myosin minimizes its ATPase activity, and thus the activity cannot be inhibited further by Ca$^{2+}$.

B. Role of actin in Ca inhibition. 1. Dephosphorylation decreases the affinity of Physarum myosin for actin. Figure 3A shows comparison of the ATPase activity of phosphorylated myosin with that of dephosphorylated myosin in the presence of various concentrations of actin. The ATPase activity of phosphorylated myosin increased with the concentration of actin. A similar increase was observed with dephosphorylated myosin, although the increase required higher actin concentrations. Kinetic analysis (inset of Fig. 3A) showed that dephosphorylated myosin had a higher $K_m$ for actin. The $V_{max}$ values were the same for both types of myosin.

Figure 3B summarizes the effect of Ca$^{2+}$ by using the extent of Ca inhibition, 100 x [(ATPase activity in EGTA) – (ATPase activity in Ca$^{2+}$)]/ (ATPase activity in EGTA), as an index. The extent of Ca-inhibition of phosphorylated myosin was high, irrespective of actin concentration. For dephosphorylated myosin, Ca-inhibition of actin-activated ATPase activity was low in the presence of low actin concentrations, and increased with the actin concentration, reaching a level comparable to that of the phosphorylated myosin. Thus, changes in the actin-activated ATPase activity associated with myosin phosphorylation can be explained by changes in the affinity of myosin for actin.

2. Ca binding mode rather than the phosphorylating mode is physiologically important. In the presence of actin at low concentrations, the ATPase activity of Physarum myosin was modified by Ca$^{2+}$ binding and by altering its phosphorylated state. However, in the presence at high actin concentrations, only Ca$^{2+}$ binding regulated the ATPase activity. Therefore, the crucial factor in determining which of the two modes is dominant in vivo is the concentration of actin in Physarum plasmodial cells. In skeletal muscles, the concentration of actin is comparable to that of myosin by weight. However, non-muscle tissues are expected to be exposed to high actin concentrations, because actin concentrations are
It is true in plasmodial cells, and hence the mode of Ca binding should be physiological. Changes in the phosphorylated state of myosin may not play a major role in vivo. The conclusion was consistent with the data shown in Table 5 that the Ca-binding activity of myosin was not affected by the phosphorylation or dephosphorylation of myosin.

VIII. Inhibitory effect of Ca$^{2+}$ on Physarum myosin as compared with the activating effect of Ca$^{2+}$ on scallop myosin

The detection of the inhibitory effect has been described so far by measuring myosin ATPase activity in the presence of actin (Fig. 1A). However, the inhibitory effect is now detected by a variety of methods (Table 6), as explained in the following paragraph.

The regulation by Ca$^{2+}$ binding to myosin was first established with myosin from the scallop adductor muscle (Fig. 4A). However, Ca$^{2+}$ activates the activity of scallop myosin, whereas it much higher than myosin concentrations. This is true in plasmodial cells, and hence the mode of Ca binding should be physiological. Changes in the phosphorylated state of myosin may not play a major role in vivo. The conclusion was consistent with the data shown in Table 5 that the Ca-binding activity of myosin was not affected by the phosphorylation or dephosphorylation of myosin.

Table 6. Calcium inhibition of actin-myosin interaction of Physarum plasmodium (modified from Ref. 20)

| Actin-activated ATPase activity of myosin$^{28,39}$ | Superprecipitation of actomyosin$^{28,39}$ (see, Section I) |
|--------------------------------------------------|-----------------------------------------------------------|
| In vitro motility assay                           | Nitella-based motility assay$^{43}$                       |
| Myosin-coated surface assay$^{47}$                | Tension development of actomyosin threads$^{48}$         |
| Contraction of cell-free model$^{49}$--$^{54}$    | (see, Section XI)                                        |
| Intact cell$^{55}$ (see, Section XI)              |                                                            |

Note that mechanical aspects of actin-myosin interaction are detected both by Nitella-based motility assay and by myosin-coated surface assay. In the former assay, latex beads about 2 µm in diameter are coated by Physarum myosin and then are introduced to the bundles of Nitella actin-filaments. The ATP-dependent movement of the beads are observed in the presence of various concentrations of Ca$^{2+}$. In the latter assay, the fluorescent actin-filaments prepared from skeletal muscle are used. They are mounted on the glass surface that is fixed by Physarum myosin. The ATP-dependent movement of actin fluorescence is observed under the fluorescent microscope.
inhibits that of Physarum myosin. Both modes were observed by measuring the actin-activated ATPase activities of the two myosins. This section describes another method for detecting the ATP-dependent sliding between actin and myosin, in which the movement of actin-filaments on myosin fixed to the surface of a coverslip was observed under a microscope. This method allows direct comparison of movement velocities in EGTA with those in Ca$_2^+$, because solutions containing EGTA and solutions containing Ca$_2^+$ can be applied sequentially to the same myosin-coated glass surface.

Physarum myosin was purified from plasmodia (Fig. 4A) by the method described in Table 2. Scallop myosin was purified from striated muscle of Patinopecten yessoensis (Fig. 4E). Actin was purified from rabbit skeletal muscle and was polymerized to form filaments. Actin filaments labeled with rhodamine-phalloidin were allowed to move on Physarum or scallop myosin fixed to a nitro-
cellulose-coated glass surface. The ATP-dependent movement of the actin filaments was monitored under a fluorescent microscope equipped with a video camera.

The mean velocity of actin movement on Physarum myosin was 0.80 µm/s in the absence of Ca²⁺ with EGTA (Fig. 4B). However, the velocity reduced to 0.31 µm/s in the presence of Ca²⁺ (Fig. 4C), which agrees with the inhibitory effect of Ca²⁺ measured by the actin-activated ATPase activity (Fig. 1A). On the scallop myosin-coated surface, the velocity was 1.28 µm/s when Ca²⁺ was present (Fig. 4C). In the presence of EGTA, almost no movement was detected (Fig. 4F). Thus, we confirmed the inhibitory effect of Ca²⁺ by comparing the mechanical movement of actin filaments for Physarum and scallop myosin. (Figs. 4D and H).⁴⁶,⁴⁷

IX. Actin-binding proteins involved in Ca inhibition of Physarum

Ca inhibition through myosin reached a maximum of about 60% (see Section VII, Fig. 3), suggesting that additional mechanisms may work for the Ca inhibition. This section describes Ca inhibition caused by modulating actin filaments in a Ca²⁺-dependent manner.

A. Fragmin. Fragmin, purified from Physarum plasmodium, bound to actin filaments at the barbed end to shorten them in the presence of Ca²⁺. This effect reduced the tension development in actomyosin threads in the presence of Ca²⁺.⁴⁸,⁵⁰ We speculated that this type of regulatory mechanism might also produce the inhibitory effect of Ca²⁺ on the actin–myosin interaction of Physarum.

B. Cytoplasmic Ca-binding light chain in Physarum plasmodial cells. In Section VI B, we explained that CaLc is a Ca²⁺-binding subunit of Physarum myosin (Table 4). However, we noticed that Physarum plasmodial cells contained CaLc that was not incorporated into myosin molecules as a light chain, and proposed that CaLc enhanced the inhibitory effect of Ca²⁺.⁴⁵,⁵⁷ Later, we found that CaLc modulates the polymerization of actin in the absence of Ca²⁺, namely CaLc polymerized actin, and this activity was abolished in the presence of Ca²⁺.⁴⁵ Because polymerized actin tends to activate myosin, we concluded that calcium light chain exerts an inhibitory effect on actin–myosin interactions through polymerized actin. Because CaLc can now be obtained as a recombinant protein (see Section X), the back-up mechanism for Ca inhibitory effects can be confirmed.

Table 7. Effect of caldesmon-like protein and calmodulin on the velocities of movement of actin filaments on coverslips coated with Physarum myosin

| Actin filament | Velocity (µm/sec, n = 30) |
|---------------|--------------------------|
| Control (0.1 mM EGTA) | 1.39 ± 0.49 |
| + Caldesmon-like protein (0.1 mM EGTA) | 1.95 ± 0.66 |
| Control (0.1 mM Ca²⁺) | 0.94 ± 0.42 |
| + Caldesmon-like protein (0.1 mM Ca²⁺) | 1.26 ± 0.60 |
| + Caldesmon-like protein + calmodulin (0.1 mM Ca²⁺) | 1.08 ± 0.50 |

C. Caldesmon-like protein. Caldesmon (CaD)-like protein was purified from Physarum (Table 7). Similar to smooth muscle CaD, it is heat-stable and has an elongated shape. It reacted with the antibody against smooth muscle CaD, and was present in Physarum cells in association with stress fibers, which was composed of actin and myosin together with their binding proteins, if any.⁴²,⁵⁸ The CaD-like protein exerted a stimulatory effect on the interaction of Physarum myosin with actin. Because the stimulation was abolished by the calmodulin in the presence of Ca²⁺, CaD-like protein enhanced the inhibitory effect on the interaction of Physarum myosin with actin. Further explanation was added in Supporting information section.

CaD was first obtained from smooth muscle, and exerted an inhibitory effect on the interaction of smooth muscle myosin; the inhibitory effect was released by calmodulin in the presence of Ca²⁺.⁶⁰ Later, Lin et al. found that the stimulatory effect was detected for smooth muscle CaD under specific conditions and that the stimulation was abolished by calmodulin in the presence of Ca²⁺.⁶¹ Because this stimulation was similar to the effect of Physarum CaD-like protein, we should re-examine how Physarum CaD-like protein produced the stimulation and its abolition.

X. Studies with recombinant Physarum myosin and its mutants

A. Expression and purification of full-length of Physarum myosin, short-tailed heavy meromyosin, and Physarum myosin with mutant Ca-binding light chain. We cloned the full-length
Physarum myosin HC (GenBank accession number AF335500), CaLc (GenBank accession number J03499; see Section VI B, Table 4), and PLc (GenBank accession number AB076705) from plasmodia, and then constructed baculovirus vectors with the wild types of HC, CaLc, and PLc. The Sf-9 cells cultured in Grace’s insect culture medium were co-infected with three constructs of HC, CaLc, and PLc simultaneously to express Physarum myosin as a recombinant protein. Using his-tagged vectors, we purified Physarum myosin by Ni-NTA Superflow column chromatography. The purified Physarum myosin consisted of 230 kDa HC, 18 kDa PLc, and 16 kDa CaLc (SDS-PAGE shown in Fig. 5A). The electron micrograph of Physarum myosin showed the structure consisting of two globular heads and a tail. The SDS-PAGE and electron micrograph results were comparable with those in Figs. 2Aa and 1D, respectively, confirming that Physarum myosin was expressed. Physarum heavy meromyosin (HMM) was expressed with HC cDNA of Met 1-Lys 118, CaLc, and PLc, followed by similar purification procedures. The SDS-PAGE and electron micrograph results were shown in Figs. 5A and B, respectively. The identification of 135 kDa HC with two globular heads was consistent with the notion that we obtained Physarum HMM as a recombinant protein.

B. Examining the role of CaLc in Ca inhibition by mutating CaLc. We engineered mutant Physarum myosin with CaLc-3A that...
showed no Ca-binding activity (Figs. 6A and B).62) We compared the effect of Ca\(^{2+}\) on wild-type *Physarum* myosin and the mutant *Physarum* myosin-3A (○), and *Physarum* HMM (▲) were expressed and purified as described in the legend to Fig. 5. A, the actin-activated ATPase activities of the three proteins were determined in 0.1 mM EGTA and the various concentrations of Ca\(^{2+}\) as expressed by pCa. B, The effect of Ca\(^{2+}\) were examined by measuring the velocity of actin-filament movement on the glass surface coated with the respective proteins.24)

**Fig. 7.** Effect of Ca\(^{2+}\) on the expressed myosin, HMM and mutant myosin. Wild type *Physarum* myosin (○), mutant *Physarum* myosin-3A (●), and *Physarum* HMM (▲) were expressed and purified as described in the legend to Fig. 5. A, the actin-activated ATPase activities of the three proteins were determined in 0.1 mM EGTA and the various concentrations of Ca\(^{2+}\) as expressed by pCa. B, The effect of Ca\(^{2+}\) were examined by measuring the velocity of actin-filament movement on the glass surface coated with the respective proteins.24)

We compared the effect of Ca\(^{2+}\) on wild-type *Physarum* myosin and the mutant *Physarum* myosin by measuring the actin-activated ATPase activity (Fig. 7A) and detecting the motor activity (Fig. 7B).22) The ATPase activity of wild-type *Physarum* myosin was reduced by increasing the Ca\(^{2+}\) concentration (filled circles of Fig. 7A), whereas the ATPase activity of the mutant *Physarum* myosin was hardly affected by the Ca\(^{2+}\) concentration (open circles of Fig. 7A). Similarly, we detected the inhibitory effect of Ca\(^{2+}\) by measuring the movement velocity of actin filaments on the surface coated with wild-type *Physarum* myosin, whereas there was no inhibitory effect on the surface coated with mutant *Physarum* myosin (Fig. 7B).

**C. Role of myosin tail length.** Electron microscopy showed that recombinant full-length myosin has a 160 nm tail (Fig. 5B1), and that HMM has a shorter tail (Fig. 5B3). The inhibitory effect of Ca\(^{2+}\) on wild-type *Physarum* myosin was more pronounced than that on HMM (filled triangles, Fig. 7), although both myosin and HMM contained wild-type CaLc. Because this difference was detectable by both ATPase activity assay (Fig. 7A) and in vitro motility assay (Fig. 7B), we concluded that the length of the HC should change the effect of Ca\(^{2+}\).

**D. Reversibility of the effect of Ca\(^{2+}\) on *Physarum* myosin.** To test the reversibility of the Ca\(^{2+}\) effect, we coated a glass coverslip surface with recombinant *Physarum* myosin, and mounted actin filaments in motility buffer containing EGTA or Ca\(^{2+}\), and subjected to the observation of the
movement of actin filaments in the following order; EGTA ⇒ Ca\(^{2+}\) ⇒ EGTA. The motility was inhibited when the motility buffer was changed from EGTA to Ca\(^{2+}\). Then, upon changing the buffer from Ca\(^{2+}\) to EGTA, the movement of actin filaments recovered (Fig. 8A). The reversible effect of Ca\(^{2+}\) was confirmed by reversing the order of the buffer (Fig. 8B).\(^{24}\) The reversibility demonstrated both ways is consistent with the idea that Ca\(^{2+}\) binding and release regulate myosin activity, which is shown in Fig. 7 of Ref. 25.

E. Role of CaLc in the inhibitory effect of Ca\(^{2+}\). To determine whether CaLc or PLc plays a more important role in exerting the inhibitory effect of Ca\(^{2+}\) on myosin, we expressed and purified HMMs associated with scallop and Physarum Lcs (Fig. 9). We used cDNA fragment coding the HC of smooth muscle myosin, because Ca\(^{2+}\) binding to and release from smooth muscle HMM were not involved in the regulation.\(^{63}\)

As shown in Fig. 9, the actin-activated ATPase activity of HMM with smooth muscle Lcs (construct #1) remained at the basal level, irrespective of the presence of Ca\(^{2+}\) or EGTA, confirming that smooth muscle Lcs are not regulated by calcium binding.\(^{63}\) We confirmed that Ca\(^{2+}\) exerted an activating effect on the recombinant HMM of scallop myosin Lcs, ScELc and ScRLc (construct #3). However, the effect of Ca\(^{2+}\) was inhibitory on the recombinant HMM of Physarum myosin Lcs, PhCaLc, and PhPLc (construct #2). For heterogeneous Lcs, HMM with ScELc and PhPLc (construct #4), and HMM with PhCaLc and ScRLc (construct #5), we observed that the actin-activated ATPase activity was increased by Ca\(^{2+}\) for scallop Lc, and that the activity was inhibited by Ca\(^{2+}\) for Physarum Lc. Therefore, the key Lc for activation was ScELc and the key Lc for inhibition was PhCaLc. In conclusion, ScELc played an important role in the activity effect of Ca\(^{2+}\) and PhCaLc for the inhibitory effect of Ca\(^{2+}\);\(^{23}\) and both are alkali Lc class (Table 3).

F. Crystal structure of the regulatory domain of Physarum myosin. CaLc has four Ca-binding sequences, which are EF-hand loops (Fig. 6A). We mutated cDNA of CaLc followed by expression in Escherichia coli cells, and then measured Ca binding to the recombinant CaLc (wild type) and mutant CaLc-3A (D15A/D17A/E26A). The Ca-binding activity of CaLc was lost in CaLc-3A (Fig. 6B). Further analysis indicated that the Ca binding site was localized in the first loop of CaLc.\(^{62}\) The neck region of the myosin molecule (Fig. 5B), known as the regulatory domain (RD), acts as a lever arm during force generation, and studies with scallop myosin have shown that it is the site of Ca\(^{2+}\) regulation. We expressed the RD of Physarum myosin, which is composed of CaLc, PLc, and a short HC fragment containing the binding site for the Lcs. RD was purified, crystallized, and its structure was determined.\(^{64}\) The scallop ELC had an extra turn in the first loop compared with Physarum CaLc, as shown in Fig. 2 of Ref. 64. Thus, we confirmed the important role of the first EF-hand loop of Physarum CaLc based on structural analysis.
XI. Inhibitory mode for Ca\(^{2+}\) regulation of contraction of \textit{Physarum} plasmodium

A. Inverse relationship of [Ca\(^{2+}\)]i with plasmodium contraction. The \textit{Physarum} plasmodial cell is a giant single cell with multiple nuclei, and it consists of a sol-like inner protoplasm and an outer layer of gel protoplasm. The sol-like protoplasm shows streaming movements of the nuclei. Actomyosin system is responsible for the contraction–relaxation movement of the outer layer.\(^{58,65}\) It is possible to measure tension development by using the strand of plasmodial cell. Yoshimoto and Kamiya treated the strand with saponin to destroy its cell membrane, and they measured the tension development by changing [Ca\(^{2+}\)]i from outside the cell.\(^{50}\) This method detected stable isotonic tension when ATP was supplied. The tension was reduced with the increase in Ca\(^{2+}\) concentration. We also measured [Ca\(^{2+}\)]i of living plasmodium and related to the contraction and relaxation of the cell. The inverse relationship of [Ca\(^{2+}\)]i with plasmodium contraction was in confirmation of the inversed relationship.\(^{55}\)

B. Detection of the inhibitory mode with actomyosin preparations. Early studies on the Ca-control of the actin-myosin-ATP interaction in \textit{Physarum} plasmodium were made with crude preparations of actomyosin. The observation that the ATPase activity of this preparation was increased with the increase in Ca\(^{2+}\) concentration led the authors to the conclusion that Ca\(^{2+}\) activated the interaction in the similar way to the regulatory way of vertebrate skeletal muscle.\(^{66}\)

We also observed that Ca\(^{2+}\) elevated the Mg-ATPase activity of crude actomyosin preparation of Table 2, Step 3 as shown by the filled circles in Fig. 10A. However, when the preparation was purified by repeated washing, the effect of Ca\(^{2+}\) on the activity was eventually reversed. As shown by the open circles in Fig. 10A, the Mg-ATPase activity of purified actomyosin (Table 2, Step 4) was inhibited with the increase in Ca\(^{2+}\) concentration. We interpreted that the reversed effect of Ca\(^{2+}\) was brought about by removing ATP pyrophosphohydrolase(s), which were Ca\(^{2+}\) activatable and abundant in plasmodium,\(^{67}\) from the crude actomyosin preparation to the purified actomyosin preparation.\(^{25}\) Because SDS-PAGE of the purified actomyosin was mainly consisted of actin and myosin (for SDS-PAGE, see Fig. 1A of Ref. 25), we reached to the conclusion that Ca\(^{2+}\) controlled the actin-myosin-ATP interaction by inhibiting actin-activated ATPase activity of myosin as reviewed in Ref. 8.

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Fig. 10. Physiological relevance of inhibitory mode of Ca\(^{2+}\) regulation. A. Mg-ATPase of crude (●) and purified (○) actomyosin. Crude actomyosin (Table 2, Step 3) and purified actomyosin (Table 2, Step 4) were prepared from the extract (Table 2, Step 2) of \textit{Physarum} plasmodium. For assay detailed conditions, see Fig. 1 of Ref. 25. B. Effect of Ca\(^{2+}\) on Mg-ATPase activity of \textit{Physarum} myosin in the presence (●) and absence (○) of skeletal muscle actin under physiological conditions (0.5 mM Mg-ATP, 1 mM Mg\(^{2+}\), 30 mM KCl, and 0.1 mM EGTA-Ca buffer at pH 6.90 and 25 °C), which were estimated from \(^{31}\)P-NMR spectrum (inset) of living plasmodia. From the spectrum, we estimated ATP concentration, free Mg\(^{2+}\) concentration and pH of living plasmodium.\(^{50}\)
C. Inhibitory mode detected in the ATPase activity of actin-activated Mg-ATPase activity of purified myosin under the physiological conditions of *Physarum* plasmodium. On a phylogenetic basis, *Physarum* belongs to a species that is quite remote from vertebrate. Although a few reports are available for the intracellular ionic concentrations, we dare to subject living plasmodium to $^{31}$P-Nuclear magnetic resonance (NMR) study. The spectrum of NMR (Insert of Fig. 10B) revealed that the intracellular pH, ATP concentration and free Mg$^{2+}$ concentration were pH 6.9, 0.2–0.5 mM, and about 1 µM, respectively.

*Physarum* myosin was purified from an actomyosin preparation as described in Section II. The ATPase activity of *Physarum* myosin activated by purified skeletal muscle actin was examined under physiological conditions. As shown in Fig. 10B, we measured the actin-activated Mg-ATPase activity of *Physarum* myosin under the physiological conditions for *Physarum* including the above data. The activity was reduced by Ca$^{2+}$ at micromolar concentrations, demonstrating that a myosin-linked inhibitory mode is present in living *Physarum* plasmodial cells.

**XII. Ca$^{2+}$-dependent phosphorylation and dephosphorylation in the *Physarum* plasmodial cell**

**A. Model of myosin signal transduction.** The phosphorylation of *Physarum* myosin at PLc and HC suggested the presence of kinases for myosin Lcs and HCs in the plasmodial cell (Section VII, Fig. 2). The removal of phosphate by an exogenous potato acid phosphatase caused the loss of the actin-activated ATPase activity of myosin$^{39}$ and the loss of phosphate content in the myosin$^{40}$ However, the phosphatase treatment did not affect the Ca$^{2+}$-binding activity of the myosin (Table 5), suggesting that the regulatory mechanism for myosin phosphorylation is independent from regulation by the Ca inhibitory mechanism. [Ca$^{2+}$]i changes periodically with the periodic changes in the direction of cytoplasmic streaming because of the actin–myosin interaction. The changes are so slow, on a time scale of minutes, suggesting that myosin phosphorylation does not play a physiological role in the plasmodial cell. As the first step in understanding the turnover of phosphate, we investigated whether the PLc of *Physarum* myosin was phosphorylated in vitro in low or high [Ca$^{2+}$]i conditions. A crude fraction containing both myosin light chain kinase (MLCK) activity and Ca$^{2+}$-binding protein was purified, and we identified the 38kDa protein as the Ca$^{2+}$-binding protein and the 55kDa protein as MLCK.$^{72}$ The effect of Ca$^{2+}$ on MLCK phosphorylation of PLc was inhibitory,$^{72,73}$ indicating that myosin is phosphorylated under low [Ca$^{2+}$]i conditions in plasmodial cells. To estimate the dephosphorylation in vivo, the actomyosin preparation (Table 2, Step 3) is an excellent model to test the regulation related to actin and myosin. The model contains the kinase activities for actin and myosin subunits and the phosphatase activity for these proteins. We incubated the preparation in $[^{32}$P] ATP to allow the kinases to phosphorylate these proteins, and the phosphorylation was terminated by the kinase inhibitor staurosporine. We observed that the radioactivity incorporated was gradually decreased by the phosphatase. The decrease in radioactivity was terminated by the phosphatase inhibitor okadaic acid, as shown in Figs. 4–6 of Ref. 73. The experiments showed that the phosphatase activity was low in the absence of Ca$^{2+}$ and increased with the increase in Ca$^{2+}$ at micromolar concentrations. Furthermore, the calmodulin inhibitor trifluoperazine inhibited the phosphatase activities, indicating that calmodulin was involved in the phosphatase activity. Thus, myosin tends to be in an active, phosphorylated form in the absence of Ca$^{2+}$, whereas it is in an inactive, dephosphorylated form in the presence of Ca$^{2+}$.

Figures 11A and B summarize the signal transduction of myosin in a plasmodial cell. Myosin is active only in the phosphorylated form, and is affected by binding Ca$^{2+}$. However, the phosphorylation of myosin does not affect the binding activity of myosin. These Ca$^{2+}$-independent pathways are shown in A by the short arrows in (1). Myosin is phosphorylated at low [Ca$^{2+}$]i in step (2) and is dephosphorylated at high [Ca$^{2+}$]i in step (3). The Ca-binding protein responsible for the phosphorylation is the 38kDa protein$^{72}$ and *Physarum* calmodulin is responsible for the dephosphorylation.$^{34}$ In B, the sites of Ca-binding (Ca$^{2+}$) and phosphorylation (❤) are schematically expressed. They are located in myosin heads, where CaLc and PLc are both located (see Section X F). Phosphorylation of PLc is a prerequisite for myosin to be in an active form. When phosphorylated myosin binds Ca$^{2+}$ at CaLc, it is inactivated as described above.

**B. Discrepancies in data on *Physarum* myosin.** Inspired by the molecular cloning of MLCK from *Dictyostelium* amoeba,$^{74}$ we used the same approach in *Physarum*. Template cDNA prepared
from *Physarum* plasmodium was subjected to the polymerase chain reaction (PCR) with degenerate PCR primers designed from MLCK and calmodulin kinases. The resulting cDNA sequence predicted a 42,650 Da protein containing 376 amino acids. The cDNA was expressed in *E. coli*, demonstrating that the *Physarum* MLCK phosphorylated PLC in the presence of calmodulin and Ca\(^{2+}\), and that Ca\(^{2+}\) stimulated *Physarum* MLCK. Thus, we obtained an MLCK that exhibited Ca\(^{2+}\)-inhibitory activity by the conventional purification procedure, and also obtained an MLCK that exhibited Ca\(^{2+}\)-stimulatory activity by molecular cloning and molecular expression. It is unknown which MLCK plays a physiological role.

A similar question arises from the difference in myosin purification techniques. *Physarum* myosin can be obtained by a biochemical purification technique (Fig. 1D) or by cDNA cloning followed by expression in cultured cells (Fig. 5B1). Indeed, the specific activity of the actin-activated Mg-ATPase of purified myosin (Fig. 1A) were similar to that of recombinant myosin (Fig. 7A of Ref. 23). For example, the actin-activated Mg-ATPase activity of biochemically purified myosin was about 150 nmol mg\(^{-1}\) min\(^{-1}\) in EGTA (Figs. 3A and 10D), and that for recombinant myosin was 160 nmol mg\(^{-1}\) min\(^{-1}\) in EGTA (Fig. 5A of Ref. 23). However, the two types of myosin showed different behavior. Purified myosin is at least partially in the phosphorylated form (Fig. 2B), and recombinant myosin is not phosphorylated. Further, Ca\(^{2+}\) inhibition was detectable in both types by measuring actin-activated ATPase activity as well as by inducing *in vitro* motility, although the phosphorylated state is quite different. The effect of phosphorylation on *Dictyostelium* myosin II differs between the PLC and HC; stimulatory effect for the former and the inhibitory effect for the latter. Although no data are available for *Physarum* myosin, the phosphorylation approach may work (see Section VIII). The discrepancy between the myosin obtained by the conventional and recombinant methods remains unresolved.

### XIII. Perspectives
The life cycle of *Physarum* consists of two phases with different modes of motility: uninucleate amoeba showing slow amoeboid movement; and multinucleated plasmodium showing rapid cytoplasmic streaming. Actomyosin was obtained from amoeba by methods similar to those used for plasmodium (Table 2) and amoebal myosin was purified from the actomyosin preparation. Plasmoidal myosin in its two-headed and long-tailed shape was examined by electron microscopy, and its subunit composition of HC and CaLc was examined by SDS-PAGE. Peptide mapping showed that the HC and PLc

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### Table

| Low [Ca\(^{2+}\)]i | High [Ca\(^{2+}\)]i |
|-------------------|-------------------|
| myosin*           | myosin-Ca\(^{2+}\) |
| Ca\(^{2+}\) Ca-binding | (1)               |
| P phosphorylation | (2)               |
| * Active          | (3)               |

**Fig. 11.** A. A proposed model of Ca\(^{2+}\) and phosphorylation. 1. Ca-binding of myosin is high (~\(\mu\)M level) irrespective of the phosphorylation as shown by the short arrow with (1). 2. Myosin is phosphorylated in low [Ca\(^{2+}\)]i as in an active form as shown by the asterisk (*). 3. The phosphate shown by the enclosed P (\(\oplus\)) is removed by the phosphatase(s) in high [Ca\(^{2+}\)]i. Note that in addition to the scheme related to myosin, the signal pathway related to actin was explained in Section IX. B. Schematic expression of myosin phosphorylation as expressed by \(\oplus\) and Ca\(^{2+}\). Myosin with two-heads and a long-tail was schematized in black (active form) or in white (inactive form).
differed between amoebal and plasmodial myosins whereas CaLc was identical. The actin-activated Mg-ATPase activities of amoebal myosin showed a similar Ca$^{2+}$-inhibitory effect to plasmodial myosin. Sonobe et al.\textsuperscript{79} prepared a contractile model by permeabilizing the membrane of \textit{Amoeba proteus}, and found that the ATP-dependent contraction inhibited by increasing Ca$^{2+}$. Based on the results from \textit{A. proteus}, it can be speculated that Ca$^{2+}$ inhibited the physiological motility of \textit{Physarum} amoeba.

A similar myosin, the conventional myosin II isoform, was purified from \textit{Dictyostelium} amoeba, and its actin-activated Mg-ATPase activity was modified by the phosphorylation mechanism. The effect of phosphorylating its Lc was stimulatory, whereas that of phosphorylating its HC was inhibitory.\textsuperscript{76} It will be intriguing to see whether the Lc or HC subunit plays a physiological role in regulating amoeba motility.

Cytoplasmic streaming, which is usually observed in plant cells, is thought to be mediated by the actomyosin system. Streaming occurs in the resting state of the cells at low Ca$^{2+}$ concentrations, and stops when the intracellular Ca$^{2+}$ concentration is increased by excitation. Ca$^{2+}$ is used as a signal for transient cessation of streaming.\textsuperscript{80} In contrast, the actomyosin system in animal cells is active only when Ca$^{2+}$ increases. As exemplified by activating muscle contractions by Ca$^{2+}$,\textsuperscript{6} Ca$^{2+}$ is an activator. Thus, the actomyosin system of \textit{Physarum}, a lower eukaryote, is akin to that of plant cells in terms of Ca$^{2+}$ regulation. \textit{Chara} is a well-characterized plant because of the rapid movement of cellular organelles along its intracellular actin-filament cables. We cloned the cDNA of the motor protein responsible for the movement.\textsuperscript{81} The structure differed from the conventional muscle myosin shown in Fig. 5B1, and was characterized as myosin XI. Myosin XI was purified biochemically and characterized from tobacco BY-2 cells. The effect of Ca$^{2+}$ on this myosin XI isoform was inhibitory, as shown by both ATPase activity and motor activity.\textsuperscript{82} Among other unconventional myosins, myosin I and myosin V were obtained from vertebrate cells by protein purification, and their motor activities were characterized. It will be intriguing to see the effect of Ca$^{2+}$ on the vesicle transportation of vertebrate cells using a similar method that was used in the study on BY-2 cells.

In this review, I proposes a question as if Ca inhibition of actomyosin activity never found in animal cell. The actomyosin system is also thought to be involved in the secretion of endocrine cells. As reported in Ref. 84, the secretion of renin from juxtaglomerular cells is inhibited by Ca. Therefore, the actomyosin systems in the cells are thought to be under inhibitory Ca control, although the conventional myosin of myosin II isoform is involved or not. I believe that more examples of such actomyosin systems will be found as the search for Ca inhibition proceeds.

**Supporting Information**

Is inhibitory effect of Ca$^{2+}$ as detected by ATPase and motility is sufficiently high enough to explain its physiological role? Ca-inhibition of the actin-activated ATPase activity of \textit{Physarum} myosin was $\sim$60% at most (Fig. 3B). As shown in Figs. 4B and C, Ca-inhibition of the actin motility on a glass surface coated with \textit{Physarum} myosin (Table 7) was

![Fig. S1. Relationship between Ca$^{2+}$ and the actin-activated ATPase activity of \textit{Physarum} myosin.\textsuperscript{42,59} (A) Myosin is regulated both by phosphorylation of PLc and by Ca$^{2+}$ binding to CaLc. Myosin must be phosphorylated to be active (-Ca$^{2+}$), and Ca$^{2+}$ inhibits the activity by binding to the myosin (+Ca$^{2+}$). (B) Caldesmon-like protein of \textit{Physarum} enhances the activity in the absence of Ca$^{2+}$ (-Ca$^{2+}$). The enhancement is abolished by calmodulin in the presence of Ca$^{2+}$ (+Ca$^{2+}$). Further, Ca$^{2+}$ (+Ca$^{2+}$) inhibits the myosin activity for caldesmon-like protein to enhance the effect of Ca$^{2+}$ (+Ca$^{2+}$).](image-url)
of Ca$^{2+}$, indicating partial effect of Ca$^{2+}$. The motility assay at the same experiment using scallop myosin, Ca-inhibition was 100% (Fig. 4FG).

To exert the inhibitory effect of Ca$^{2+}$ to Physarum myosin, the myosin must be activated in advance as described in Section VII. Phosphorylation of the myosin at PLC is responsible for the activation (Fig. 11B). Indeed, Ca$^{2+}$ exerts the inhibitory effect on the myosin activated in this way. Figure S1A relates schematically myosin activity to Ca$^{2+}$ concentration. The activity is high in the absence of Ca$^{2+}$ (−Ca$^{2+}$), which is caused by myosin phosphorylation. Upon addition of Ca$^{2+}$, myosin binds Ca$^{2+}$, and the activity is inhibited (+Ca$^{2+}$).

The caldesmon-like protein of Physarum binds to actin and stimulates the actin-activated ATPase activity of Physarum myosin and the actin motility. Stimulation is abolished when calmodulin is mixed with the caldesmon-like protein in the present of Ca$^{2+}$. Figure S1B schematically explains that the caldesmon-like protein enhances additionally the activity of myosin that is activated by phosphorylation in the absence of Ca$^{2+}$ (−Ca$^{2+}$). Upon mixing calmodulin and Ca$^{2+}$, calmodulin abolishes the enhancement through actin to cause Ca-inhibition (+Ca$^{2+}$). In addition to Ca-inhibition of myosin itself, we can detect larger Ca-inhibition. Such an actin-linked mode may back up the inhibitory effect of Ca$^{2+}$ on Physarum myosin, resulting in the augmentation of Ca-inhibition. As explained in Section IX of the text, a few actin-binding proteins including caldesmon-like protein are found in Physarum plasmodia. They may contribute to the augmentation of the effect.

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Profile

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