**135. Ca^{2+} -binding Light Chain of *Physarum* Myosin Confers
Inhibitory Ca^{2+} -sensitivity on Actin-myosin-ATP
Interaction via Actin^{†)}**

By Kazuhiro KOHAMA,^{*)} Taro Q. P. UYEDA,^{**)}
Hiromi TAKANO-OHMURO,^{***)} Takeshi TANAKA,^{†)}
Toshikazu YAMAGUCHI,⁺⁺⁾ Kei MARUYAMA,^{*)}
and Tomoko KOHAMA^{*)}

(Communicated by Setsuro EBASHI, M. J. A., Dec. 12, 1985)

Introduction. We have shown that Ca^{2+} inhibits actin-myosin-ATP interaction of *Physarum* plasmodium by inhibiting the interaction,^{1)–8)} in sharp contrast with the activating effect of Ca^{2+} on the interaction of muscle,⁹⁾ and proposed that this is the mechanism underlying the control of the cell motility of the plasmodium.

Under physiological conditions, which are estimated from the result of ^{31}P -nuclear magnetic resonance of living plasmodia,¹⁰⁾ the inhibitory Ca^{2+} -control is mediated by Ca^{2+} -binding to myosin.⁷⁾ However, this myosin-linked regulation is hardly detectable under high Mg^{2+} conditions.^{2),4),6),7)}

We isolated a fraction containing Ca^{2+} -dependent inhibitory factor from myosin B or natural actomyosin of *Physarum*.²⁾ The addition of this fraction to actomyosin reconstituted of pure actin and myosin showed Ca^{2+} -inhibition under high Mg^{2+} conditions.²⁾ As the factor bound to actin, the factor seemed to exert its regulatory action via actin.²⁾ However, the active principle(s) has not yet been identified.

Under physiological conditions, myosin B is more sensitive to Ca^{2+} than reconstituted actomyosin.⁶⁾ As myosin B contains both myosin and Ca^{2+} -dependent inhibitory factor, regulatory actions of Ca^{2+} on myosin B are mediated by both actin and myosin, i.e., *Physarum* actomyosin system is subjected to dual regulation.⁶⁾

In this paper, we isolate a fraction enriched with Ca^{2+} -binding myosin light chain of 14,000 Mr, the fraction which confers the inhibitory Ca^{2+} -sensitivity on the actin-myosin-ATP interaction under high Mg^{2+} -conditions. This 14,000 Mr component, seemingly identical with the Ca^{2+} -binding light chain, is considered as Ca^{2+} -dependent inhibitory factor.

Materials and methods. Myosin was prepared from plasmodia of *Physarum*

^{†)} This work was supported in part by grants from Yamada Science Foundation and the Aino Hospital Foundation and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

^{*)} Department of Pharmacology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113.

^{**)} Department of Biology, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113.

^{***)} Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113.

^{†)} Research division, Saitama Red Cross Blood Center, Yono 338.

⁺⁺⁾ Biomedical Laboratories, Kawagoe 350.

polycephalum.⁶⁾ A fraction enriched with Ca^{2+} -binding light chain (see Results) of 14,000 Mr was prepared from myosin by denaturing myosin heavy chain and then subjecting to ammonium sulfate fractionation.⁷⁾ Actin was prepared from rabbit skeletal muscle.^{11),12)} The purity of protein preparations was monitored by SDS polyacrylamide gel electrophoresis (SDS PAGE).¹³⁾

^{125}I -labeling of the Ca^{2+} -binding light chain was carried out in Bolton and Hunter reagent (Amersham). Actin-affinity column was prepared by conjugating F-actin with Ultrogel resin.¹⁴⁾ $^{45}\text{Ca}^{2+}$ -binding to the 14,000 Mr light chain was demonstrated on Western blot of SDS PAGE of myosin.¹⁵⁾ The ATPase activity of myosin was measured using a pH stat at pH 7.50 at 25°C .¹⁶⁾ The Ca^{2+} -concentration was calculated using apparent binding constant for Ca/EGTA of $2.5 \times 10^7 \text{ M}^{-1}$ at this pH.^{17),18)} Protein concentrations were determined by the method of Lowry using bovine serum albumin as a standard.¹⁹⁾

A polyclonal antibody against the Ca^{2+} -binding light chain was raised with guinea pigs by injecting the light chain, which was obtained by purifying a fraction enriched with the light chain by the preparative SDS PAGE. A monoclonal antibody against the myosin heavy chain was produced by hybridoma formation²⁰⁾ using BALB/c mice immunized with the heavy chain purified by the preparative SDS PAGE of myosin.²¹⁾ The young plasmodia just after differentiation from amoebae of the colonial strain¹⁰⁾ were subjected to double staining with the antibodies against the light and heavy chains.²²⁾ Further details will be described elsewhere.

Results. The Mg-ATPase activity of *Physarum* myosin was clearly inhibited by Ca^{2+} in the presence of skeletal muscle actin (Table I, upper line), confirming the previous results⁴⁾⁻⁷⁾ that Ca^{2+} exerts its inhibitory effect through myosin.

Table I. Effect of Ca^{2+} -binding myosin light chain on Ca^{2+} -sensitivity of actomyosin reconstituted of *Physarum* myosin and skeletal muscle actin

light chain $\mu\text{g/ml}$	Mg^{2+} mM	KCl mM	ATPase activity		
			(n mol/min/mg myosin)		%*
			EGTA	50 μM Ca^{2+}	
0	3.5	20	79.3	28.0	64.7
0	8.5	5	119.5	97.6	18.3
3.5	8.5	5	123.2	61.0	50.5

* $100 \times (\text{ATPase in EGTA} - \text{ATPase in } \text{Ca}^{2+}) / (\text{ATPase in EGTA})$. Actin-activated ATPase activities were determined in the solutions containing 1.5 mM ATP, 100 $\mu\text{g/ml}$ of actin, 41 $\mu\text{g/ml}$ of myosin, 0.1 mM Ca-EGTA buffer and specified concentrations of the light chain, Mg^{2+} and KCl shown in the table.

We examined which subunit of myosin should be responsible for its high affinity for Ca^{2+} ; ^{45}Ca radioactivity was detected in the 14,000 Mr band of Western blot of myosin (Fig. 1). This observation is compatible with that of Kessler *et al.*²³⁾ and suggests that Ca^{2+} exerts its inhibitory action by binding to this Ca^{2+} -binding light chain.²³⁾

The inhibitory effect of Ca^{2+} on the actin-activated ATPase activity of myosin was hardly observed when assayed in the presence of high Mg^{2+} concentrations (Table I, middle line) in accord with the previous results.^{2),4),6),7)} However, the inhibitory effect of Ca^{2+} was restored upon addition of a fraction enriched with the Ca^{2+} -binding light chains (Table I, lower line).

Fig. 1. SDS PAGE of a fraction enriched with 14,000 Mr light chain and ⁴⁵Ca-autoradiograph of Western blot of myosin. a: Western blot of SDS PAGE of myosin was stained by amidoblack. b: The Western blot was allowed to react with a solution containing ⁴⁵Ca and subjected to autoradiography.¹⁵⁾ c: SDS PAGE of a fraction enriched with 14,000 Mr light chain stained with Coomassie brilliant blue.

The fraction was labeled by ¹²⁵I and absorbed to the actin-affinity column at low ionic strength. The radioactivity was eluted from the column with a solution of a high ionic strength (Fig. 2). This observation strongly suggests that the Ca²⁺-binding light chain binds also to actin.

The antibody against myosin heavy chain stained only peripheral region of the plasmodial cell (Fig. 4b), but that against Ca²⁺-binding light chain, whose specificity was tested in Fig. 3, stained the whole cell (Fig. 4a). These observations indicate that a considerable part of the Ca²⁺-binding light chain is not incorporated into myosin molecules.

Thus it is quite possible that, in addition to the Ca²⁺-binding light chain incorporated into myosin, myosin B may contain another kind of Ca²⁺-binding light chain which works as a Ca²⁺-dependent inhibitory factor through actin. The latter light chain may be responsible for the Ca²⁺-sensitivity of myosin B which

Fig. 2

Fig. 2. Binding of the Ca^{2+} -binding light chain to actin-affinity column. A fraction enriched with the Ca^{2+} -binding light chain was labeled with ^{125}I and applied to the actin-affinity column equilibrated with the binding buffer consisting of 10 mM KCl, 3.5 mM Mg acetate, 20 mM Tris-HCl pH 7.5 and 0.2 mM DTT. The column was washed with the binding buffer exhaustively. Then the elution buffer consisted of 0.5 M KCl, 3.5 mM Mg acetate, 20 mM Tris HCl, 0.2 mM DTT and 0.2 mM EGTA was applied to the column at the position indicated by an arrow. Aliquots of the eluate from the column were subjected to gamma-counting of ^{125}I . The ionic strength of the eluate was monitored by measuring conductivity.

Fig. 3. Immunoblots of myosin subunits.²¹⁾ SDS PAGE of myosin (a) was subjected to immunoblots using antibodies against myosin heavy (b) and Ca^{2+} -binding light (c) chains. Hp, heavy chain; L18p, 18,000 Mr light chain; L14, Ca^{2+} -binding light chain of 14,000 Mr.

Fig. 3

is higher than that of reconstituted actomyosin.⁶⁾

Discussion. This paper suggests that *Physarum* myosin B contains additional Ca^{2+} -binding light chain which is not incorporated into myosin. The Ca^{2+} -dependent inhibitory factor, which confers inhibitory Ca^{2+} -sensitivity on actin-myosin-ATP interaction by binding to actin, was isolated from myosin B preparation by mild treatment that may not compel myosin to release its light chain.⁷⁾ Thus the active principle appears to be the light chain itself which was contained in myosin B preparation but not in the myosin molecules.

Fig. 4. Double staining of *Physarum* plasmodium with antibodies against the Ca²⁺-binding light chain (a), and myosin heavy chain (b).

So far the Ca²⁺-control of actin-myosin-ATP interaction, whether it would be inhibitory¹⁾⁻⁸⁾ or excitatory,^{9),24)} has been classified in two categories, i.e., myosin-linked²⁴⁾ and actin-linked.⁹⁾ The myosin-linked control seems responsible for the inhibitory Ca²⁺-sensitivity of the interaction of *Physarum*.⁴⁾⁻⁸⁾ The present study has revealed that the Ca²⁺-binding light chain exerts its regulatory action also through actin as a Ca²⁺-dependent inhibitory factor. Thus the Ca²⁺-control of this lower eukaryote assumes a new type of regulation, in which a single small Ca²⁺-binding protein exhibits a dual control through both actin and myosin.

Acknowledgements. We thank Prof. Y. Nonomura and M. Furuya for their discussions, interest and support. Our thanks are also due to Prof. S. Ebashi, M. J. A., for reading the manuscript.

References

- 1) Kohama, K. *et al.* (1980): Proc. Japan Acad., **56B**, 591-596.
- 2) Kohama, K. (1981): J. Biochem., **93**, 1829-1832.
- 3) Ebashi, S. *et al.* (1982): Calcium Modulators (eds. Godfraind, T. *et al.*). Elsevier, pp. 39-49.
- 4) Kohama, K., and Kendrick-Jones, J. (1982): J. Muscle Res. Cell Motility, **3**, 491 (abstr.).
- 5) Kohama, K. *et al.* (1983): Europ. J. Cell Biol., **suppl. 1**, 25 (abstr.).
- 6) Kohama, K., and Kohama, T. (1984): Proc. Japan Acad., **60B**, 435-439.
- 7) Kohama, K. (1985): Cell Motility, Mechanism and Regulation (eds. Hatano, S. *et al.*). University of Tokyo Press, pp. 65-76.
- 8) Kohama, K., and Shimmen, T. (1985): Protoplasma, **129**, 88-91.
- 9) Ebashi, E., and Endo, M. (1968): Progr. Biophys. Molec. Biol., **18**, 123-183.
- 10) Kohama, K. *et al.* (1984): FEBS Lett., **176**, 161-165.
- 11) Kohama, K. (1980): J. Biochem., **87**, 997-999.
- 12) Kendrick-Jones, J. *et al.* (1976): J. Mol. Biol., **104**, 747-775.
- 13) Laemmli, U. K. (1970): Nature, **227**, 680-685.
- 14) Mikawa, T. (1980): (cited in ref. 2)).
- 15) Maruyama, K. *et al.* (1983): J. Biochem., **131**, 1-15.
- 16) White, H. D. (1982): Methods in Enzymol., **85B**, 698-708.
- 17) Saïda, K., and Nonomura, Y. (1978): J. Gen. Physiol., **72**, 1-14.
- 18) Harafuji, H., and Ogawa, Y. (1980): J. Biochem., **87**, 1305-1312.
- 19) Lowry, O. H. *et al.* (1951): J. Biol. Chem., **193**, 265-275.
- 20) Galfre, G. *et al.* (1978): Nature, **266**, 550-552.
- 21) Takano-Ohmuro, H. *et al.* (1985): J. Cell Biol., **100**, 2025-2030.
- 22) Uyeda, T. Q. P., and Furuya, M. (1985): Protoplasma, **126**, 221-232.
- 23) Kessler, D. *et al.* (1980): Cell Motility, **1**, 63-71.
- 24) Kendrick-Jones, J., and Scholey, J. M. (1981): J. Muscle Res. Cell Motility, **2**, 347-372.