THE EFFECT OF MYOSIN ANTIBODY ON THE DIVISION OF STARFISH BLASTOMERES

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ABSTRACT

Antiserum against starfish egg myosin was produced in rabbits. Antibody specificity to myosin was demonstrated by Ouchterlony's immunodiffusion test and by immunoelectrophoresis in the presence of sodium dodecylsulfate (SDS). The latter technique showed that the antibody binds to both heavy and light chains of egg myosin. Furthermore, the antibody reacted with starfish sperm myosin and starfish adult muscle myosin at both the heavy and light chains. It did not react with bovine platelet myosin or rabbit skeletal muscle myosin in Ouchterlony's test; however, a weak reaction was observed in the presence of SDS between the antibody and these myosin heavy chains. Ca- and Mg-ATPase activities of egg myosin were not affected by the antibody, but it did inhibit actin-activated ATPase activity of egg myosin. Microinjection of the antibody into blastomeres of starfish eggs at the two-cell stage was carried out. Anti-egg myosin γ-globulin inhibited the subsequent cleavages at an amount of more than 0.3 ng when injected at interphase. The inhibition was reduced when the injection was carried out near the initiation of cleavage. At the onset of the second cleavage the antibody was not inhibitory; however, an appropriate amount inhibited the third cleavage. Although the disappearance of the nuclear membrane was observed in the presence of the antibody, the formation of the mitotic apparatus was more or less disturbed. However, the formation of daughter nuclei seemed to be scarcely affected by the antibody except that the distance between the nuclei was significantly smaller than normal.

The cortical gel contraction theory of cytokinesis first proposed by Marsland and Landau (22) has been confirmed experimentally by several workers (2, 11, 30) who demonstrated that measurable tension is exerted at the cleavage furrow. The discovery by Schroeder and by others that the "contractile ring" in the cleavage furrow of various dividing cells is composed of numerous microfilaments (1, 4, 38–40, 42, 43, 45, 46, 48) strongly supported this idea. The force exerted by the cleavage furrow of various sea urchin and sand dollar eggs has been estimated to be 1-3 × 10^5 dyn/cm^2, a value comparable to the tension developed in skeletal muscle (14, 30). Actually, the microfilaments in the contractile ring have been identified as actin filaments in newt eggs (27), in crane fly spermatocytes (6), and in HeLa cells (41) by means of decoration with heavy meromyosin (15). The fluorescent heavy meromyosin technique has also demonstrated that actin is accumulated in the cleavage furrow during cell division of HeLa cells (35).

Although myosin filaments have not yet been found in the contractile ring, we have recently

isolated myosin from the isolated cortical layer of dividing sea urchin (18) and starfish eggs (19). From these observations it would be reasonable to suspect that actin and myosin would interact to produce the force of constriction. To test this possibility, we produced antibodies against starfish egg myosin. These antibodies, when microinjected into starfish eggs, inhibit cleavage.

MATERIALS AND METHODS

Purification of Myosins

The starfish, *Asterias amurensis* Lütken, was obtained from both Akkeshi Bay and Tokyo Bay. The species was identified by Dr. R. Hayashi, Emeritus Professor of Toyama University. Eggs were collected and inseminated as described previously (19).

Starfish egg myosin and crude fractions of egg myosin, i.e., 0–35% and 35–55% saturated ammonium sulfate fractions from the high-speed supernate of the egg extract and 0.2 M KCl-precipitates of the 35–55% ammonium sulfate fractions, were prepared as described previously (20). Starfish sperm myosin was prepared as described previously (21). Bovine platelet myosin was prepared as described elsewhere (44). Rabbit skeletal muscle myosin and actin were prepared as described by Perry (28) and by Mommaerts (25), respectively.

Muscle myosin from the tube feet of adult starfish was purified by the following method. Tube feet were detached from the starfish by raking off with a spatula; they were washed once with chilled ordinary sea water and then washed once with 1 M glycerol, 10 mM $NaHCO_3$ (pH 7.8), and stored in 50% (vol/vol) glycerol, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithiothreitol (DTT), 10 mM Sorensen's phosphate buffer (pH 7.0) at −20°C. After being stored for 1 mo, they were washed with 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM DTT, 10 mM phosphate buffer at pH 7.0 (buffer A), and homogenized in buffer A with a motor-driven Teflon-glass homogenizer for one to two strokes. Myofibrils were released from the tube foot ghosts by this procedure. The homogenates were centrifuged at 2,000 *g* for 5 min. The sediment consisted of two layers; the bottom layer containing the tube foot ghosts, the upper layer consisting mostly of myofibrils. The latter was isolated and washed twice with buffer A. To this suspension of myofibrils was added enough 3 M KCl, 50 mM phosphate (pH 7.0) to give a final KCl concentration of 0.6 M. The suspension was stirred for 1 h and then centrifuged at 40,000 *g* for 10 min. 10 vol of chilled water were added to the supernate, and the flocculent precipitate formed was collected at 10,000 *g* for 10 min, dissolved in and dialyzed against 0.6 M KCl, 10 mM phosphate, 0.2 mM DTT at pH 7.0. This fraction was designated as the tube foot actomyosin fraction. To the actomyosin solution, enough 0.4 M ATP (pH 7.0), 0.1 M sodium pyrophosphate (Na-PPi) (pH 7.0) and 0.1 M $MgCl_2$ were added to give final concentrations of 10 mM, 2 mM, and 1 mM, respectively. The solution was then centrifuged at 260,000 *g* for 2 h to sediment the actin. The materials precipitated from the supernate at 35–55% saturation with respect to ammonium sulfate were collected. This fractionation step removed both a paramyosin-like protein and the remaining actin. The ammonium sulfate fraction was subjected to gel filtration with a Sepharose 4B column (2.5 × 90 cm) eluting with 0.6 M KCl, 0.5 mM ATP, 0.5 mM $MgCl_2$, 0.1 mM DTT, 10 mM Tris·HCl (pH 7.4) as described previously (20). ATPase activity of each fraction was measured, and the faster eluting half of the ATPase peak was pooled, concentrated by precipitation at 55% saturation of ammonium sulfate, dissolved in 0.6 M KCl and further precipitated in 70 mM KCl, 0.5 mM DTT and 5 mM phosphate buffer at pH 7.0. This fraction was designated as the tube foot myosin fraction. The slower eluting half of the myosin peak was contaminated by a high molecular weight protein (see Results).

Preparation of Antisera and γ-Globulin Fraction

1 mg of egg myosin from *Asterias amurensis* obtained at Akkeshi Bay was dialyzed against 0.15 M NaCl, 10 mM phosphate buffer at pH 7.0, and emulsified with Freund's complete adjuvant. This solution was injected subcutaneously into the back of a male New Zealand white rabbit. After 2 wk, about 20 ml of blood was obtained by ear bleeding. The second injection was carried out as described above 4 wk after the first injection. About 3 wk after the second injection, the second bleeding was carried out (50 ml each at intervals of several days). Serum was separated from plasma clots and treated at 56°C for 30 min. Antiserum from the first or the second bleeding was called the first or the second antiserum, respectively. Preimmune serum was taken before immunization and used in control experiments. The γ-globulin fraction was obtained from the serum by an ammonium sulfate cut of 20–33% saturation; it was dissolved in 0.15 M KCl, 10 mM phosphate buffer at pH 7.0.

Immunodiffusion and Immunoelectrophoresis

Ouchterlony's immunodiffusion tests were carried out in a 1% agarose gel containing 0.6 M KCl and 10 mM $Na\text{-}PP_i$ (pH 7.2). The well volume was 30 μl.

Immunoelectrophoresis was carried out according to Obinata et al. (26) with some modifications: a 4% agarose gel plate (7.4 × 8.5 cm) containing 0.05% SDS; 0.1 M Tris, 0.4 M boric acid (pH 7.3) was used. The applicability of immunoelectrophoresis with antiserum (not fractionated antibody)[1] in the presence of dilute SDS was

[1] After Masaki (personal communication), fractionated γ-globulin or IgG is not applicable to this technique. A possible "chelating effect" of albumin on SDS to protect antibodies from SDS is considered.

first shown by Masaki (23). Antigen fractions were previously dialyzed against 8 M urea, 0.5% SDS, 0.1 M β-mercaptoethanol, 5 mM EDTA, 20 mM Tris·HCl (pH 8.5) overnight and then against 0.05% SDS, 0.1 M Tris, 0.4 M boric acid, 5 mM DTT for 2 h. Electrophoresis was carried out at 200 v for about 2 h with bromphenol blue as a tracking dye. Duplicates were always run. After electrophoresis, one plate was immediately stained with 0.04% Amido black dissolved in 10% (vol/vol) acetic acid, 40% (vol/vol) methanol to assure the position of migrated protein subunits. The other plate was used for immunodiffusion. Antisera were placed in narrow troughs (1 mm-wide) made on the gel and the plate was left standing at 4°C. Two days were sufficient to obtain complete immunoprecipitation.

Analytical Methods

Disc electrophoresis in the presence of SDS was carried out with a Tris-glycine system (18) in 4% or 5% acrylamide gels polymerized with ammonium persulfate.

ATPase activities were assayed at 25°C as described previously (20). In brief, Ca-ATPase activity was assayed in 0.6 M KCl, 5 mM $CaCl_2$, 1 mM ATP and 10 mM piperazine-*N,N'*-bis (2-ethanesulfonic acid)·NaOH buffer (pH 6.5). Actin-activated ATPase activity was assayed in 75 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, and 20 mM Tris·maleate buffer (pH 7.0).

Protein concentration was determined by the procedure of Lowry et al. (17), using bovine serum albumin as a standard.

Turbidity of a solution was measured at 660 nm, using a Hitachi 101 spectrophotometer.

Microinjection of Antibody into the Living Egg

Fertilized eggs of *Asterias amurensis* were cultured for 1.5 h in a petri dish at 20 ± 1°C. At various stages before the onset of the second cleavage, one of the blastomeres was microinjected with anti-egg myosin γ-globulin fraction from the second antiserum or the preimmune γ-globulin fraction, while the other blastomere served as a control. The method of microinjection was fundamentally the same as Hiramoto's (13, 33). The concentration of the γ-globulin fraction microinjected was 27–53 mg/ml. The volume of the solution injected was 0.4–3.0 × 10^{-8} ml which corresponds to 0.8–6% of the cell volume.

To determine the diffusibility of the injected protein in the cytoplasm; fluorescein isothiocyanate-labeled goat IgG (FITC-IgG) was injected into the cell and its diffusion was observed under a Nikon FL fluorescence microscope.

Chemicals and Biochemicals

The chemicals and their sources are as follows: ATP (Kyowa Hakko Kogyo, Co., Tokyo, Japan), agarose (Agarose Pure, Koch-Light Laboratories, Ltd., Colnbrook, Bucks, Eng.), Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.), FITC-IgG (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.), SDS (Wako Pure Chemical Co., Tokyo, Japan). Other reagents used were all of analytical grade. Water was deionized and glass-distilled.

RESULTS

Disc Gel Electrophoresis of Myosins from Several Sources

The electrophoretic pattern of *Asterias* egg myosin is shown in Fig. 1*a*. As described previously (20), it consists of a 210,000-dalton heavy chain and two classes of light chains of about 20,000 dalton. The *Asterias* sperm myosin fraction (Fig. 1*b*) has similar bands but also contains impurities because the gel filtration step was omitted in the purification procedure (21). Bovine platelet myosin (Fig. 1*c*) and rabbit skeletal muscle myosin (Fig. 1*d*) fractions revealed protein bands consistent with those of earlier reports (16, 29, 37).

An actomyosin fraction prepared from the tube feet of *Asterias* contains as major components (Fig. 1*e*) two high molecular weight proteins (one about 300,000 dalton), a myosin heavy chain, a paramyosin-like protein (110,000 dalton), a 55,000-dalton protein, actin, and myosin light chains. After purification, the tube foot myosin fraction consists mainly of the heavy chain and two classes of light chains (Fig. 1*f*). The 300,000-dalton high molecular weight protein runs slower than myosin in the agarose gel column and, therefore, could be purified (Fig. 1*g*). ATPase activity was not detected in this fraction, and properties of the protein were not investigated further in this work.

From the co-electrophoresis experiments on 4% acrylamide gels, it was found that the heavy chains of *Asterias* egg myosin, sperm myosin, tube foot myosin, and bovine platelet myosin co-migrated with one another with a mobility slower than that of rabbit skeletal muscle myosin.

Properties of Antibody against Asterias Egg Myosin

Ouchterlony's immunodiffusion tests were carried out applying 0.6, 0.8, or 1.1 mg egg myosin per milliliter in the central well. A single precipitin line was observed in all cases and was detectable even if the antiserum from the second bleeding (3 wk after the second injection; the second antiserum) was diluted to 1/32 and egg myosin applied in

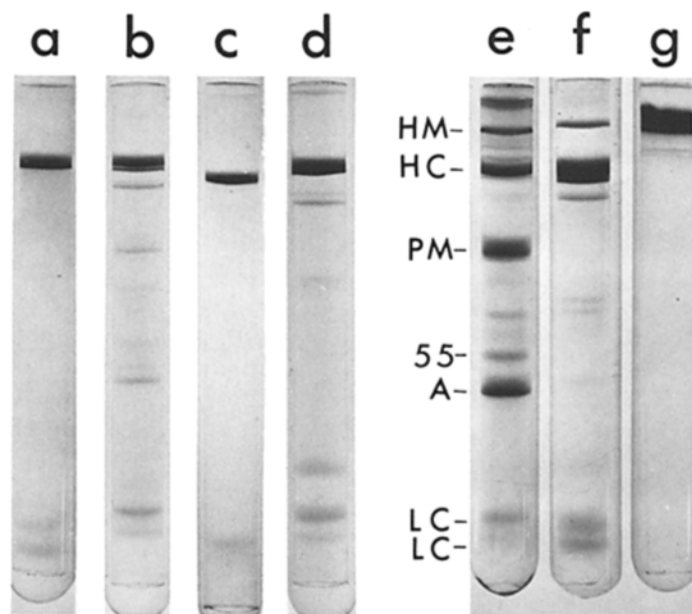


FIGURE 1 SDS-gel electrophoresis of myosins. (*a*) *Asterias* egg myosin, 11 μg; (*b*) *Asterias* sperm myosin, 20 μg; (*c*) bovine platelet myosin, 10 μg; (*d*) rabbit skeletal muscle myosin, 13 μg; (*e*) *Asterias* tube foot actomyosin, 41 μg; (*f*) *Asterias* tube foot myosin, 30 μg; (*g*) *Asterias* tube foot high-molecular weight protein, 23 μg. Electrophoresis was carried out on 5% acrylamide gels in a Tris-glycine system. *HM:* 300,000-dalton molecular weight protein. *HC:* myosin heavy chain, *PM:* paramyosin-like protein. *55:* 55,000-dalton molecular weight protein. *A:* actin. *L:* light chain.

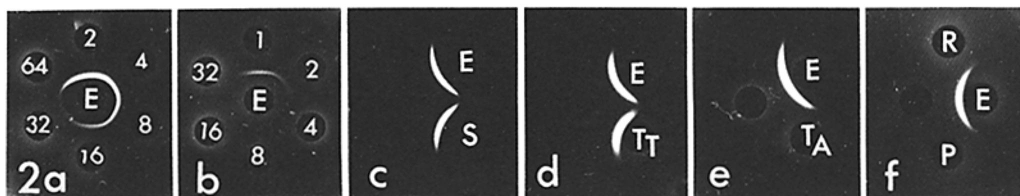


FIGURE 2 Ouchterlony's immunodiffusion tests. These tests were carried out in 1% agarose containing 0.6 M KCl, 10 mM Na-PPi at pH 7.2. (*a*) The peripheral wells contain the second antiserum with serial two-fold dilutions. The numbers indicated on the wells are the dilution index. *E:* egg myosin, 0.6 mg/ml. (*b*) The peripheral wells contain the first antiserum with serial two-fold dilutions. *E:* egg myosin, 1.1 mg/ml. (*c*) The left well contains the two-fold diluted second antiserum. *E:* egg myosin, 0.8 mg/ml. *S:* sperm myosin, 1.2 mg/ml. (*d*) The left well contains the undiluted second antiserum. *E:* egg myosin, 0.8 mg/ml. T_T: tube foot myosin from Tokyo Bay *Asterias,* 1.1 mg/ml. (*e*) The left well contains the undiluted second antiserum. *E:* egg myosin, 0.8 mg/ml. T_A: tube foot myosin from Akkeshi Bay *Asterias,* 1.5 mg/ml. (*f*) The left well contains the two-fold diluted second antiserum. *E:* egg myosin, 0.8 mg/ml. *R:* rabbit skeletal muscle myosin, 1.3 mg/ml. *P:* bovine platelet myosin, 1.2 mg/ml.

a concentration of 0.6 mg/ml (Fig. 2*a*). On the other hand, the antiserum isolated 2 wk after the first injection (the first antiserum) no longer showed a precipitin line when diluted to 1/8 with 1.1 mg/ml of egg myosin applied (Fig. 2*b*).

Immunoelectrophoresis in the presence of SDS showed that the second antiserum contained antibodies against both the heavy and light chains (Fig. 3*a*). We could not estimate whether both the light chains or one of the two light chains reacted with the antibodies because the separation of the light chains in 4% agarose gel was not clear. However, the first antiserum contained antibodies only to the heavy chain (Fig. 3*b*). The second

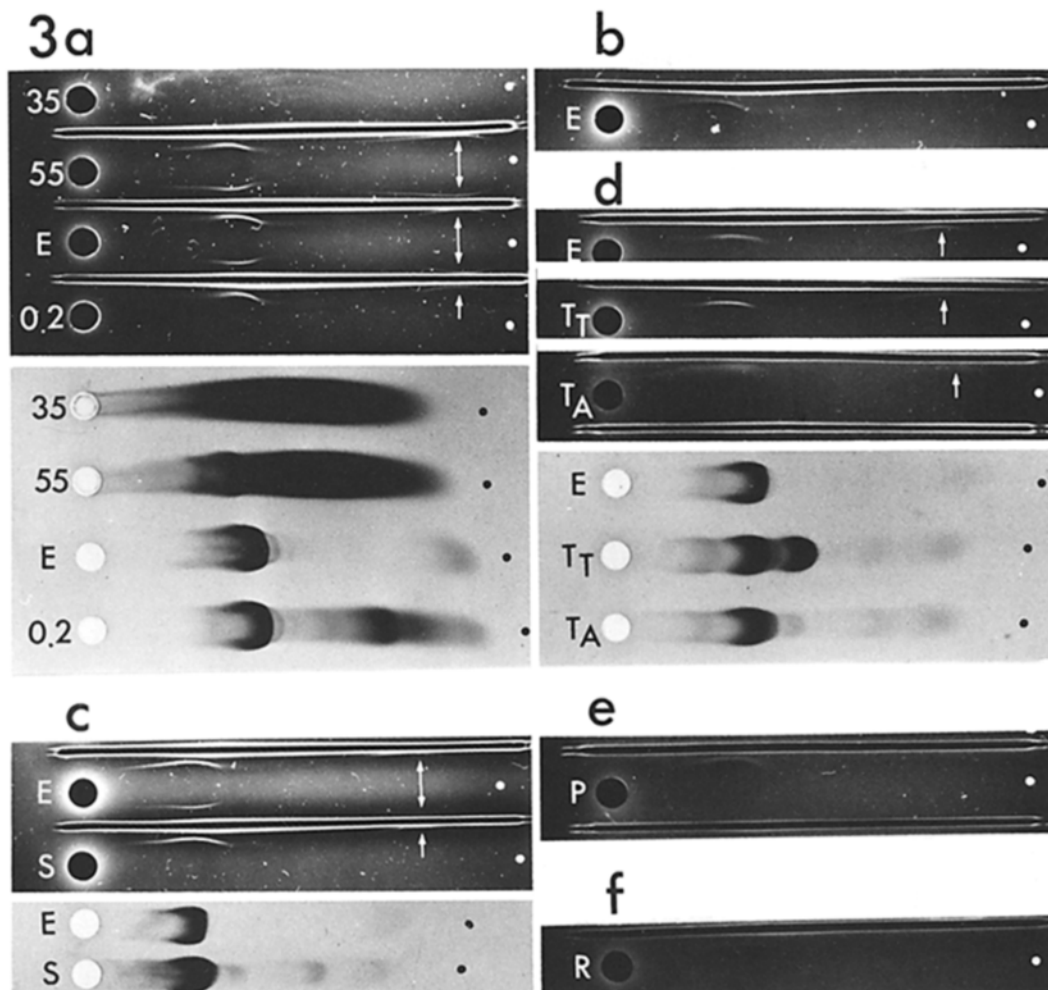


FIGURE 3 Immunoelectrophoresis experiments. Electrophoresis was carried out in 4% agarose containing 0.05% SDS, 0.1 M Tris, 0.4 M boric acid buffer, pH 7.3 (for other details, see Materials and Methods). White or black dots in the right side of the photographs indicate the front. Arrows indicate precipitin lines formed between the light chains and the antibody. (*a*) *35:* 0–35% saturated ammonium sulfate fraction, 11.2 mg/ml; *55:* 35–55% saturated ammonium sulfate fraction, 9.5 mg/ml; *E:* egg myosin, 1.0 mg/ml; 0.2: 0.2 M KCl precipitates of the 35–55% ammonium sulfate fraction, 1.9 mg/ml. All troughs contain the second antiserum. Precipitin lines are seen at the positions of the heavy and light chains. The lower picture shows the electrophoretic patterns. (*b*) *E:* egg myosin, 1.0 mg/ml. The trough contains the first antiserum. A precipitin line is seen at the heavy chain position. (*c*) *E:* egg myosin, 0.8 mg/ml; *S:* sperm myosin, 1.2 mg/ml. The troughs contain the second antiserum. Precipitin lines are seen at the positions of the heavy and light chains. The lower picture shows the electrophoretic patterns. (*d*) *E:* egg myosin, 0.8 mg/ml; T_T: tube foot myosin from the Tokyo Bay *Asterias*, 2.4 mg/ml; T_A: tube foot myosin from the Akkeshi Bay *Asterias*, 1.5 mg/ml. The upper three troughs contain the second antiserum whereas the lowest trough contains the preimmune serum. Precipitin lines are seen at the positions of the heavy and light chains near the antiserum troughs. The lower picture shows the electrophoretic patterns. (*e*) *P:* bovine platelet myosin, 1.2 mg/ml. The upper trough contains the second antiserum whereas the lower trough contains the preimmune serum. A weak precipitin line is seen at the heavy chain position near the antiserum trough. (*f*) *R:* rabbit skeletal muscle myosin, 1.3 mg/ml; The trough contains the second antiserum. A weak precipitin line is seen at the heavy chain position.

antiserum did not contain antibodies to proteins other than egg myosin. This was confirmed when immunoelectrophoresis was carried out with crude egg myosin fractions, i.e., 0-35% and 35-55% saturated ammonium sulfate fractions and 0.2 M KCl precipitates (Fig. 3*a*). In addition, myosin could not be detected in the 0-35% saturated ammonium sulfate fraction by this method. The second antiserum did not contain antibodies to other muscle proteins which was revealed by immunoelectrophoresis of the tube foot actomyosin fraction (not shown).

For the reasons mentioned above, the second antiserum was used in the experiments described below. Anti-egg myosin reacted with sperm myosin in a manner similar to egg myosin both by Ouchterlony's test (Fig. 2*c*) and by immunoelectrophoresis (Fig. 3*c*). The precipitin lines in the Ouchterlony's test fused with each other when the antigen wells were placed closer (not shown). The reaction between the anti-egg myosin and tube foot myosin was curious. The antibody against egg myosin of Akkeshi Bay *Asterias* reacted with the tube foot myosin of Tokyo Bay *Asterias* in Ouchterlony's test (Fig. 2*d*) and in immunoelectrophoresis (Fig. 3*d*) as it did in the case of sperm myosin. The latter experiment showed both the heavy and light chains to be reactive. However, no precipitin line was observed between the tube foot myosin of Akkeshi Bay *Asterias* and anti-egg myosin (same starfish) in the Ouchterlony's test (Fig. 2e). In the presence of SDS, both chains became reactive but the precipitin lines that formed were weaker than those with tube foot myosin of Tokyo Bay *Asterias* (Fig. 3*d*). A somewhat similar result was obtained with bovine platelet myosin or rabbit skeletal muscle myosin. These myosins did not react with the antibody in the Ouchterlony's test (Fig. 2*f*), but formed a weak precipitin line with the antibody only at the heavy chain position in the immunoelectrophoresis (Figs. 3*e* and 3*f*). These precipitin lines were not of nonspecific precipitation since no line was observed between the heavy chains and preimmune serum (Figs. 3*d* and 3*e*).

Effect of Anti-Egg Myosin on the Egg Myosin ATPase Activity

When the antiserum was added to the egg myosin solution in 0.6 M KCl, the turbidity of the solution increased with increasing amounts of added antiserum (Fig. 4). The amount of precipitated egg myosin was determined by measuring the Ca-ATPase activity of the clarified supernatant. 25 μg of egg myosin were precipitated by 75 μl of the antiserum (Fig. 4). The antibody against egg myosin did not seem to affect the Ca-ATPase activity. Even when all myosin molecules were covered by the antibodies to form precipitable complexes, more than 90% of the activity remained (Fig. 4).

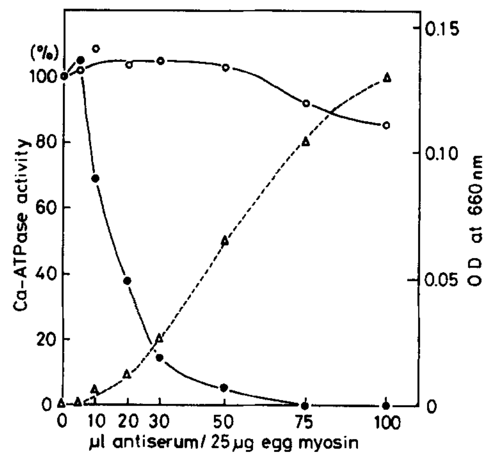


FIGURE 4 The effect of anti-egg myosin serum on the Ca-ATPase activity of egg myosin. 25 μg of egg myosin were mixed with the second antiserum to make a final volume of 1.0 ml which contains 0.6 KCl, 10 mM piperazine-*N*,*N*'-bis (2-ethanesulfonic acid) · NaOH (pH 6.5). △: turbidity (OD at 660 nm) of the mixture of egg myosin and the antiserum. (○): Ca-ATPase activity of the mixture. (●): Ca-ATPase activity of the supernatant of the mixture obtained by centrifugation at 40,000 *g* for 10 min.

The effect of the antibody on actin-myosin interactions was studied by examining whether or not anti-egg myosin γ-globulin fraction inhibits actin-activated egg myosin ATPase activity. As shown in Table I, actin-activated egg myosin ATPase activity was inhibited with increasing γ-globulin fraction of the antiserum and the inhibition was complete at a γ-globulin: egg myosin weight ratio of 400:8. The γ-globulin fraction from preimmune serum did not inhibit the actin-activated ATPase activity significantly. The antibody had no effect on the Mg-ATPase activity of egg myosin in the absence of actin (Table I).

Effect of Anti-Egg Myosin on Cell Division In Vivo

In order to determine the diffusibility of proteins injected into the cell, fluorescent proteins

Table I

Effect of Anti-Egg Myosin on Actin-Activated Egg Myosin ATPase Activity

Components for ATPase assay	Mg-ATPase activity	Actin-activated ATPase activity*	Inhibition
	nmol Pi/min/mg egg myosin		%
Egg myosin	6.1		
+ anti-myosin γ-globulin, 1.65 mg	5.8		
+ anti-myosin γ-globulin, 4.13 mg	6.2		
+ actin	27.4	21.3	
+ actin + preimmune γ-globulin, 3.00 mg	25.7	19.6	8.0
+ actin + anti-myosin γ-globulin, 0.22 mg	23.6	17.5	17.0
+ actin + anti-myosin γ-globulin, 0.83 mg	15.9	9.8	54.0
+ actin + anti-myosin γ-globulin, 1.65 mg	11.0	4.9	77.0
+ actin + anti-myosin γ-globulin, 4.13 mg	6.1	0	100

Assay conditions: egg myosin, 80 μg; rabbit skeletal muscle actin, 293 μg; 76.5 mM KCl, 1mM ATP, 1 mM $MgCl_2$, 20 mM Tris·maleate (pH 7.0); 25°C.

* Difference between the activities in the presence and absence of actin.

were microinjected into a starfish blastomere at the two-cell stage. As shown in Fig. 5, the microinjected FITC-IgG diffused into the entire cytoplasm after about 1 min. Therefore, it seems likely that there was no problem in the diffusion of the γ-globulin injected. A detailed analysis on the diffusion of proteins in the cytoplasm of marine eggs will be presented elsewhere.[2]

Under the experimental conditions employed in this study, eggs completed the first cell division 2 h after fertilization. The blastomeres in which the interphase nuclei were clearly recognized were microinjected. The duration of the interphase between the first and second divisions, where interphase nuclei were visible, was about 40 min. The second division took place 15 min after the disappearance of the nuclear membrane.

The results of the microinjection experiments are summarized in Table II. Microinjection of preimmune γ-globulin fraction had no effect on cell division at any stage tested and with any quantity of the injected proteins tested (Fig. 6), except that division was retarded by about 10 min as compared with the noninjected blastomere (Fig. 6*c*). This delay seemed to be caused by the injurious effect of the microinjection because a similar delay was observed when 0.15 M KCl, 10 mM phosphate was injected.

[2] Okuno, M., in preparation.

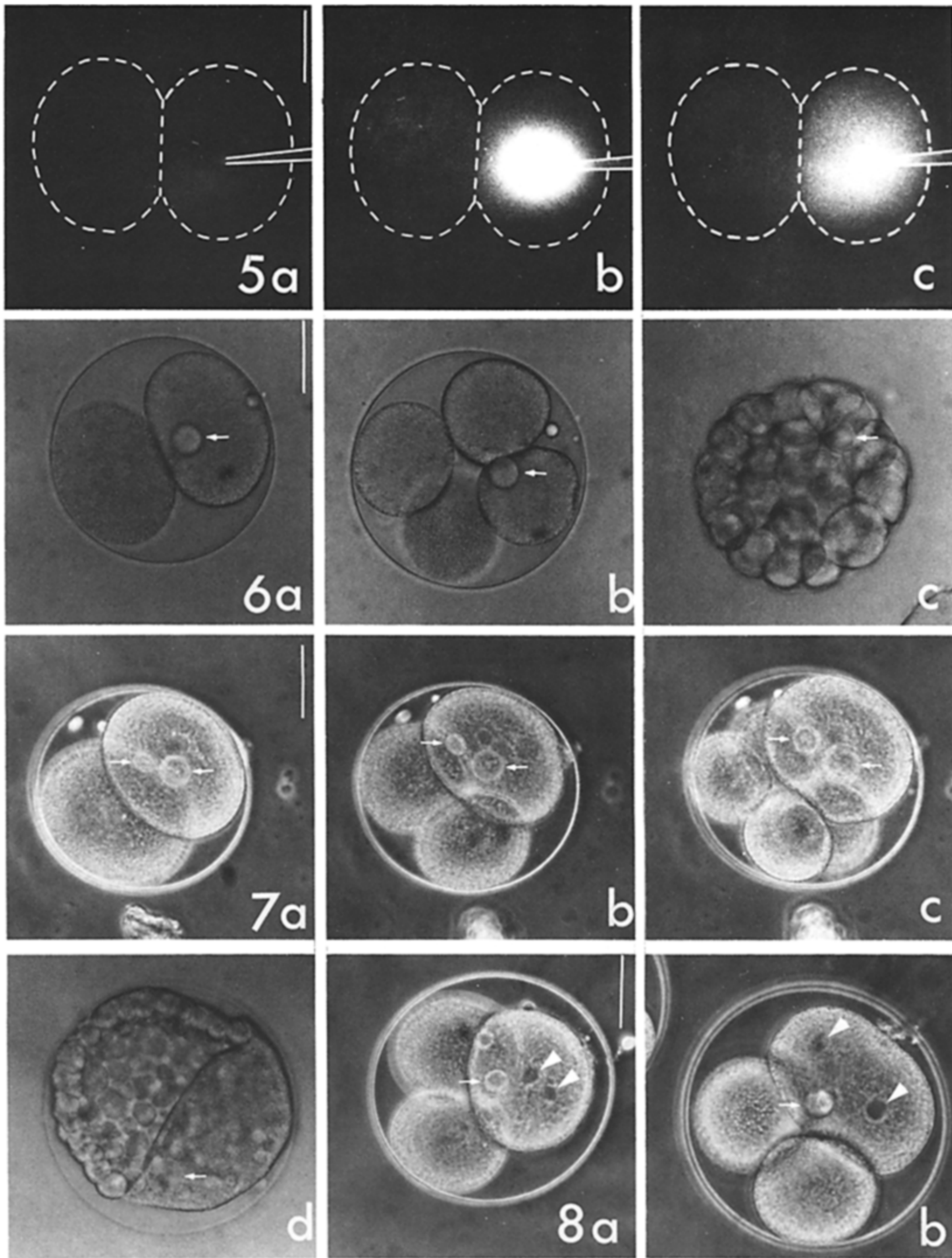
Microinjection of more than 0.3 ng of anti-egg myosin γ-globulin at interphase inhibited the second cleavage furrow formation as well as subsequent ones (Fig. 7). Only one out of 24 cells tested formed a furrow. In this cell the furrow soon regressed, however. The inhibitory effect of less than 0.2 ng of γ-globulin was incomplete. After the disappearance of the nuclear membrane, the quantity of anti-egg myosin γ-globulin that is necessary to inhibit cleavage in about half of the cells increased to around 0.4–0.5 ng. When it was injected just before furrowing or at the onset of cleavage, the cleavage furrow rapidly regressed in a small number of cells. In a majority of cells, however, the amount of antibody used in this experiment (up to 0.5 ng) did not prevent cleavage. In these experiments, when the amount of injected γ-globulin was below 0.2–0.3 ng, the third cleavage also occurred, but more γ-globulin prevented the third cleavage even though it did not prevent the second cleavage (data not shown).

In cases where anti-egg myosin γ-globulin failed to prevent cleavage, nuclear division was normal. Likewise, normal nuclear divisions were observed when a preimmune γ-globulin fraction was injected. The effect of the antibody on nuclear division was investigated in 17 blastomeres which failed to cleave following antibody injection before the dissolution of the nuclear membrane. In the presence of the antibody, dissolution of the nuclear membrane occurred in all the cells after a brief delay (about 10 min). Five cells did not develop further and did not form asters. The 12 remaining cells formed a mitotic apparatus. This apparatus was smaller and more obscure than that of control cells, however. Formation of daughter nuclei was observed in 9 out of the 12 cells (Fig. 8*a*). The center-to-center distance between the daughter nuclei was 20–30 μm. In control cells or cells arrested (Fig. 8*b*) by antibody injection after the disappearance of the nuclear membrane, the separation of the daughter nuclei was about 60 μm.

DISCUSSION

Properties of the Antibody against Starfish Egg Myosin

In *Asterias amurensis* from Akkeshi Bay the antibody against egg myosin did not react with the tube foot muscle myosin in 0.6 M KCl. This result may be interpreted as indicating that the cytoplasmic myosin differs from muscle myosin in this


5a
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species. Sperm myosin from the same starfish reacted with the antibody either in 0.6 M KCl or in 0.05% SDS. In the latter experiment, the antibody reacted with both the heavy chain and the light chains of sperm myosin as in the case of egg myosin. Moreover, there was no difference in the serological properties between the Tokyo and the Akkeshi preparations of gamete myosin. However, tube foot myosin from the Tokyo Bay *Asterias* had the same serological properties as egg myosin of the starfish from Akkeshi Bay. Such a difference between these tube foot myosins in different populations of the same species is difficult to explain at present.

The reactions between the antibody against egg myosin and the Akkeshi tube foot myosin, bovine platelet myosin or rabbit skeletal muscle myosin are intriguing as no reaction was observed in double diffusion on agarose containing 0.6 M KCl, yet in the presence of SDS the heavy chain reacted with the antibody. Since the heavy chain is a large polypeptide, there may be many antigenic sites. It is possible that the antigenic sites of the heavy chains of these myosins, which are similar to those of egg myosin, are not localized on the surface of these heavy chains but inside of them and are masked in 0.6 M KCl and unmasked by SDS. The weakness of the precipitin line when compared with that formed by the egg myosin-antibody complex might be due to the fact that these antigenic sites, similar to those of egg myosin, are few in these myosin heavy chains.

The Ca- and Mg-ATPase activities of egg myosin were not affected by the binding of the antibodies. This means that the enzymatically active regions of these molecules are not covered by the antibodies. However, since the actin-egg myosin interaction was prevented by the antibodies, these antibodies must attach to sites near the active centers, thereby sterically inhibiting the

FIGURE 5 Diffusion of FITC-IgG within the cell. Photographs were taken at an exposure of 5 s. The outline of the cell and the micropipette are shown by the white broken lines. 1.5×10^{-8} ml of FITC-IgG solution (10 mg/ml) were injected into one of the blastomeres 30 min after the onset of the first cleavage. (*a*) before injection, (*b*) 5 s after injection, (*c*) 65 s after injection. Scale bar, 50 μm.

FIGURES 6–8 The effects of γ-globulins on cell division. The scales indicate 50 μm. The arrowheads indicate nuclei. The arrows indicate silicon oil drops injected to mark experimental cells.

FIGURE 6 Cleavage and early development of the embryo after injection of preimmune γ-globulins. (*a*) 0.53 ng of preimmune γ-globulin fraction was injected into one of the blastomeres 20 min before the onset of the second cleavage. (*b*) Same egg as in (*a*), 5 min after the onset of cleavage. (*c*) 5 h after fertilization. 0.54 ng of preimmune γ-globulin fraction was injected 37 min before the onset of the second cleavage. Both blastomeres developed normally but each cleavage of the injected blastomere occurred a little later than that of a noninjected one; the division cycles of the cells on the right of the embryo, derived from the injected blastomere, are retarded several minutes behind the cells on the left of the embryo.

FIGURE 7 Cleavage and early development of the embryo after injection of anti-egg myosin γ-globulins. In Fig. *a-c*, a phase contrast microscope was used. (*a*) 0.52 ng of anti-egg myosin γ-globulin fraction was injected 16 min before the onset of the second cleavage of the control cell. (*b*) The same egg as in a, 13 min after the onset of the second cleavage of the control cell. The control cell divided into two, but the injected cell did not cleave. (*c*) The same egg as in (*a*), 55 min after the onset of the second cleavage of the control cell. The control cell divided into four, but the injected cell did not cleave. (*d*) 8 h after fertilization. 0.30 ng of anti-egg myosin γ-globulin fraction was injected 28 min before the onset of the second cleavage of the control cell. The control cell developed normally forming a blastocoel, but the injected cell never cleaved.

FIGURE 8 The nuclear division of cleavage-arrested cells. Observations were carried out by phase contrast microscopy. (*a*) This photograph was taken 30 min after the onset of the second cleavage of the control cell. 0.51 ng of anti-egg myosin γ-globulin fraction was injected 25 min before the onset of the second cleavage of the control cell. The injected cell did not cleave, but the nuclear division occurred forming daughter nuclei (see arrowheads). The distance between centers of the nuclei is 20 μm. (*b*) This photograph was taken 27 min after the onset of the second cleavage of the control cell. 0.40 ng of anti-egg myosin γ-globulin fraction was injected at metaphase 8 min before the onset of the second cleavage of the control cell. The distance between daughter nuclei is 60 μm (see arrowheads). The shape of the injected cell was slightly elongated but the cell did not cleave.

TABLE II

Effect of Injected γ-Globulins on Cleavage of Starfish Blastomeres

	Amount of injected proteins					
Anti-Egg myosin γ-globulin	($\times 10^{-1}$ *ng*)					
Stage of microinjection	1	2	3	4	5	13
Interphase						
C*	5	6	0	0	0	
F	0	0	0	1	0	
N	2	6	11	3	9	
Nuclear membrane disappeared						
C	2		9	2	4	
F	1		0	1	1	
N	0		2	4	3	
Onset of cleavage						
C	2	1		2	7	
F	0	0		1	2	
N	0	0		1	0	
Preimmune γ-globulin						
Interphase						
C			5		5	3
F			0		0	0
N			0		0	1
Nuclear membrane disappeared						
C			7		8	1
F			0		0	0
N			1		1	0
Onset of cleavage						
C			4			
F			0			
N			0			

* C, cleavage occurred. F, cleavage furrow formed but regressed. N, cleavage furrow was not formed.

binding of actin but not interfering with the association of small molecules such as ATP with the active centers.

Effect of Antibody on Cell Division In Vivo

More than 0.3 ng of anti-egg myosin γ-globulin inhibited cleavage furrow formation when injected at interphase. After the disappearance of the nuclear membrane, it was still capable of preventing cleavage although the inhibitory power was lessened. From these experiments, it is reasonable to conclude that myosin is involved in cytokinesis.

The egg myosin content of an *Asterias* egg cortex at metaphase-anaphase of the first division has been estimated to be 0.58×10^8 molecules (19), which corresponds to 0.05 ng. Assuming that the egg myosin is localized mainly in the cortical layer at this stage and that during division each daughter cell receives half the myosin molecules, the weight ratio of injected γ-globulin (0.3 ng) to egg myosin is about 10:1. From Table I, about half of the actin-activated ATPase activity of egg myosin was suppressed by about 10 times the amount of anti-egg myosin γ-globulin. This value seems to be in good agreement with the amount necessary to inhibit cleavage in vivo.

In a recent report (8), antibodies prepared against myosin were shown to be localized in the cleavage furrow as well as in the polar regions of the mitotic apparatus of human cells. The presence of myosin in the furrow region is consistent with the concept that the mechanism of cytokinesis involves interactions between actin and myosin. Thus, antibodies against myosin molecules inhibit cleavage by interacting with a major component of the contractile ring.

There are four different ways by which the myosin antibody could prevent cleavage. (*a*) It has been reported (31) that damage to the mitotic apparatus prevents formation of the cleavage furrow; or if the asters are reduced by chemical treatment of sea urchin eggs, these eggs are then unable to cleave. In our experiments we also observed that the mitotic apparatus was smaller than normal when the antibody was injected at interphase (this will be discussed in detail later). Thus, the antibody may directly interfere with cleavage furrow formation by interacting with the mitotic apparatus. However, we also observed that the antibody prevented cleavage in about half of the cells although the mitotic apparatus was of normal size (see Fig. 8*b*) when the antibody was injected after disappearance of the nuclear membrane. Thus, this possibility for the effect of the antibody on cleavage may be eliminated. (*b*) The antibody may prevent cleavage by interfering with the signal (cleavage stimulus) which is transmitted from the mitotic apparatus to the equatorial cortical layer to induce cleavage furrow formation (10, 32). The signal may not be a diffusible substance since it must accumulate in the narrow equatorial region. If it were calcium ions (47), they might be translocated in a membrane-bound or protein-bound form. If the signal is attacked by the antibody, it may consist of myosin aggregates (see below) which are to be incorporated into the contractile ring. There is no evidence for or against such a possibility.

(*c*) The antibody binds and inactivates myosin molecules when they are in the cytoplasm. In an

earlier study (20), egg myosin formed small filamentous aggregates (5–10 nm wide and 0.16 μm long) at an ionic strength of 0.4 and bipolar thick filaments (10–15 nm wide and about 0.25 μm long) at an ionic strength of 0.35. Therefore, even if the intracellular ionic strength of marine eggs were rather high, egg myosin might be in an aggregated state in vivo, although these filaments would be indistinguishable from actin filaments as seen by electron microscopy of thin sections. Fujiwara and Pollard's observation (8) that myosin is accumulated in the cleavage furrow suggests the possibility that these filaments might move from the deeper cytoplasm to the equatorial cortical layer during mitosis. The antibody may attack the myosin molecules before or during the accumulation. Even if these myosin filaments could subsequently accumulate at the equatorial cortical layer, they could not function since the antibody attached to myosin molecules would inhibit the actin-myosin interaction.

(*d*) The antibody attacks myosin molecules in the established contractile ring to stop their function. However, this possibility may be excluded since the antibody was less or ineffective when injected just before cleavage or after initiation of furrow formation when, at least in the latter case, the contractile ring is already formed (40). This result could, however, be explained by assuming that the structure of the contractile ring itself or the surrounding cytoplasmic gel layer protects myosin filaments from the invasion of the antibodies.

It has recently been reported that actin (3, 5, 7, 9, 10, 36) and myosin (8, 24) are present in the mitotic or meiotic spindles. However, the function of these proteins in the spindle is not known. Our experiments showed that anti-egg myosin might affect nuclear division to some extent. Five cells out of 17 did not form asters, and the mitotic apparatus formed in the rest of the cells was small and obscure. This mean that the formation of the mitotic apparatus was more or less disturbed by the antibody. Once the mitotic apparatus was formed in the presence of the antibody, the majority of the cells formed daughter nuclei. This result suggests that the antibody has little effect on chromosomal separation and nuclear membrane formation. The closeness of the daughter nuclei formed might be due to the fact that the mitotic apparatus formed was smaller than that in normal cells. The idea that myosin may not be involved in chromosomal separation is supported by the results reported by Sakai et al. (34) who have shown that chromosome motion in vitro induced by Mg-ATP in the isolated mitotic apparatus of sea urchin or starfish eggs was not inhibited by egg myosin antibody but inhibited by sperm flagellar dynein antibody.

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