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ACTIN AND MYOSIN A FROM LEUCOCYTES

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SUMMARY

1. An acetone powder of actomyosin was prepared from equine leucocytes and an actin fraction was obtained from it.

On addition of 0.1 M KCl to this actin fraction, the viscosity of the mixture gradually increased. Electron micrographs of the fraction showed helical, filamentous structures with a pitch and width of about 35 nm and 7.5 nm, respectively. The appearance of the fraction was similar to that of skeletal F-actin. In the presence of Mg^{2+} at low ionic strength a mixture of this fraction and skeletal myosin A showed typical superprecipitation on addition of ATP. From these results, the preparation was concluded to be leucocytic actin.

2. Myosin B from equine leucocytes was dissolved in a solution containing ATP, Mg^{2+} and EGTA at high ionic strength and centrifuged. The myosin A fraction of leucocytes was isolated from the resultant supernatant by precipitation with 45 to 55% saturation of $(NH_4)_2SO_4$. The ATPase activity of the fraction was enhanced by Ca^{2+} and inhibited by Mg^{2+}, that is, it showed the myosin A type of ATPase activity. The Ca^{2+}-dependent ATPase activity was higher at high ionic strength than that at low ionic strength. This character was similar to that of myosin A from smooth muscle. At an ionic strength of less than 0.1, the appearance of this fraction under the electron microscope was similar to that of myosin aggregates of smooth muscle, with a diameter of about 15 nm. The length of the aggregates was less than that of skeletal myosin A aggregates. A mixture of this fraction and skeletal F-actin showed remarkable superprecipitation on addition of ATP and Mg^{2+} at low ionic strength. From these results the fraction is concluded to be leucocytic myosin A.

3. A mixture of leucocytic F-actin and myosin A showed typical superprecipitation in the presence of ATP and Mg^{2+} at low ionic strength. The Mg^{2+}-dependent ATPase activity of the leucocytic myosin A was enhanced by addition of F-actin. At low ionic strength, the ATPase activity of the mixture was activated by Ca^{2+} and Mg^{2+}, and at high ionic strength, it was inhibited by Mg^{2+} and activated by Ca^{2+}, that is, it showed actomyosin-type ATPase activity. The extent of the superprecipitation of the mixture was higher at high concentration of Mg^{2+} than at low concentration. These effects of Mg^{2+} indicate that synthetic actomyosin of leucocytes is similar to that of smooth muscle.

4. From above findings it is concluded that the contractile protein of leucocytes, like smooth muscular actomyosin, is composed of actin and myosin A as elementary substances.

INTRODUCTION

From studies on the motility of leucocytes we suggested in 1954 (ref. 1) that the movement of leucocytes must be due to an actomyosin-like contractile protein in them. In support of this idea, in 1968 we succeeded in isolating an actomyosin-like protein from equine leucocytes[2]. We also isolated a contractile protein with Ca^{2+} sensitivity, that is natural actomyosin, from the leucocytes[3]. Under the electron microscope, at high ionic strength these isolated proteins had characteristic arrow head structures, like those of preparations from skeletal muscles. The proteins gave a clearing response on addition of a high concentration of ATP and Mg^{2+} at low ionic strength and dissociated thick and thin filamentous structures were seen by electron microscope like those of well-defined contractile protein from skeletal muscle[4]. Ultracentrifugal analysis of these proteins in solutions of high ionic strength containing ATP and Mg^{2+} showed that they were composed of two components with sedimentation coefficient of 5.5–6.5 S and 27–32 S, respectively.

The above evidence suggests that the contractile protein in leucocytes is a conjugate of myosin A and actin like that of muscle.

This paper reports to confirm this possibility by separate isolation of myosin A and actin from leucocytes and presents some characteristics of the myosin A and the actin from leucocytes in comparison with those from skeletal muscle.

MATERIALS AND METHODS

Actin and myosin A were isolated from actomyosin or natural actomyosin from equine leucocytes as reported previously[2,3].

Isolation of actin from leucocytes

Actomyosin was dissolved in 0.6 M KCl, diluted with 3 mM cysteine solution (adjusted to pH 7.0 with Tris) and adjusted to an ionic strength of 0.05. The resultant precipitate was washed thoroughly with 0.4% $NaHCO_3$ and then washed twice with ice-cold water and 3 times with acetone; it was then dried. 1 g of this acetone powder of actomyosin was thoroughly mixed with 50 ml of 3 mM cysteine solution containing 1 mM ATP (adjusted to pH 7.0 with Tris) and the mixture was agitated gently for 1 h at 0 °C. The insoluble material was removed by centrifugation at $1000 \times g$ for 30 min. The supernatant was subjected to isoelectric precipitation by adjusting its pH to 4.7 with 1 M acetic acid. The resulting precipitate was dissolved in a small amount of 3 mM cysteine with 1 mM ATP (pH 7.0) and the solution was dialysed against 3 mM cysteine and 0.05 mM ATP in 1 mM Tris–maleate buffer (pH 8.2) for 48 h, and then centrifuged at $1 \cdot 10^4 \times g$ for 10 min. The resulting supernatant was stirred for 1 h with 0.1 M KCl and 2 mM $MgCl_2$ at room temperature to polymerize G-actin to F-actin. During this treatment the solution became viscous. The mixture was centrigufed at $10 \cdot 10^4 \times g$ for 150 min. The precipitate was dissolved with a small

amount of 3 mM cysteine with 1 mM ATP (pH 8.2) and dialysed against 1 mM Tris–maleate buffer (pH 8.2) for 15 h to remove cations and convert F-actin to G-actin. Then the mixture was centrifuged at $10 \cdot 10^4 \times g$ for 90 min. The resulting supernatant was again stirred with 0.1 M KCl for 2 h at room temperature, and centrifuged at $10 \cdot 10^4 \times g$ for 2 h to precipitate pure F-actin. The precipitate was dissolved in 0.05 mM ATP in 3 mM cysteine (pH 8.2) and 0.1 M KCl and used as the preparation of F-actin of leucocytes.

Extraction of myosin A from leucocytes

Actomyosin is known to dissociate into myosin A and actin at high ionic strength in the presence of Mg^{2+} and ATP. Thus, to extract myosin A from leucocytic contractile protein, final concentrations of 10 mM ATP, $MgCl_2$ and EGTA and 2 mM dithiothreitol were added to a solution of the natural actomyosin of leucocytes in 0.6 M KCl (about 5 mg/ml). The mixture was adjusted to pH 8.0 with KOH and centrifuged at $10 \cdot 10^4 \times g$ for 2 h. The supernatant was dialysed against 3 mM histidine buffer (pH 7.0) with 0.5 M KCl and treated with $(NH_4)_2SO_4$ and material precipitated at 45 to 55% saturation was again dialysed against 3 mM histidine buffer (pH 7.0) containing 0.5 M KCl and 2 mM dithiothreitol, and then centrifuged at $10 \cdot 10^4 \times g$ for 2 h. The supernatant was used as the preparation of myosin A.

Preparation of skeletal actin

Skeletal actin was prepared from skeletal muscle from the back of a rabbit by the method of Katz and Hall[5]. To isolate G-actin, the procedure was carried out at 4 °C to avoid contamination with native tropomyosin. G-actin was treated with 0.1 M KCl to convert it to F-actin.

Isolation of skeletal myosin A

Protein was isolated from skeletal muscle from the back of a rabbit by the method of Perry[6]. This protein did not show any superprecipitation or decrease in viscosity on addition of ATP, proving that the preparation was not contaminated with actin.

Superprecipitation

Superprecipitation was measured turbidometrically at 660 nm in a Carry 14 spectrophotometer, using the procedure of Ebashi[7].

Determination of ATPase activity

ATPase activity was determined by measuring the amount of inorganic phosphate liberated from ATP by Marsh's method[8]. The reaction was initiated by addition of the substrate, ATP.

Determination of protein content

Protein was measured by the method of Lowry *et al.*[9], using bovine serum albumin as standard.

Ultracentrifugation

Sedimentation velocity was determined in a Hitachi VCA-1 analytical ultracentrifuge.

Amino acid analysis

Test protein was hydrolysed in a sealed tube by treatment with 6 M HCl at 110 °C for 22 h and the hydrolysate was analysed in a Hitachi, KLA-3B, automatic amino acid analyser.

Determination of viscosity

Viscosity was determined with an Ostwald's viscosimeter, of 1 ml capacity, at 25 °C.

Electron micrographs

Micrographs of actin and myosin A were taken by the negative staining technique of Huxley[10]. One drop of ice-cold preparation was placed on a microgrid coated with collodion carbon. The preparation was negatively stained with 1% uranyl acetate, and examined in a Nihon Denshi, Model 100-B, electron microscope.

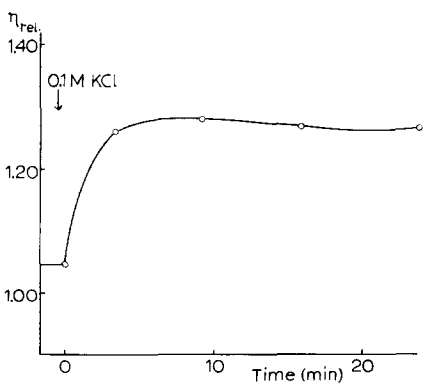
Redistilled water was employed to prepare solutions and all chemicals used were of analytical grade.

RESULTS

Nature of F-actin from leucocytes

On addition of univalent cations, G-actin of skeletal muscle polymerizes and is converted to F-actin and during this conversion the viscosity of the solution increases. We obtained a similar result using actin from leucocytes (Fig. 1).

On ultracentrifugation, the preparation gave a single peak with an $s_{20,w}$ value of about 3.2 S (Fig. 2). This value is virtually identical to 3.25 S and 3.85 S of the sedimentation coefficient of G-actins from skeletal muscle[11] and sheep uterine smooth muscle[12], respectively.



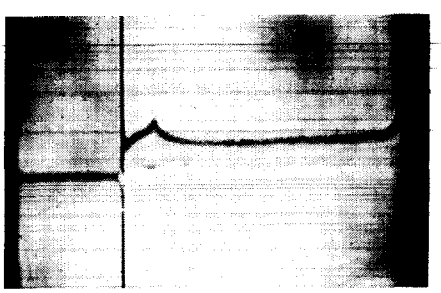


Fig. 1. Viscosity change of leucocyte actin on addition of KCl to the final concentration of 0.1 M. Reaction mixture: 0.31 mg protein/ml, 3 mM cysteine, 0.05 mM ATP and 1 mM Tris–maleate buffer (pH 8.2) at 20 °C.

Fig. 2. Sedimentation pattern of leucocyte actin. Protein concentration, 1.2 mg/ml. Other additions: 3 mM cysteine and 0.05 mM ATP. Ultracentrifugation was carried out at 51 200 rev./min at 24 °C at an angle 70°. The photo was taken 32 min after reaching maximal speed. $s_{20,w} = 3.2$ S.

The amino acid composition of G-actin from leucocytes is shown in Table I in comparison with those of skeletal muscle[25] and uterine smooth muscle[12]. The amino acid composition of the protein showed no major differences from skeletal muscle[25] and from uterine smooth muscle[12], except that the content of glutamic acid and proline in leucocytic actin was about 1.4-fold higher than those in the others.

TABLE I

AMINO ACID COMPOSITION OF ACTIN AND MYOSIN A FROM SKELETAL MUSCLE, SMOOTH MUSCLE OR LEUCOCYTES

Values for amino acids are residue in moles/10^5 g protein.

Amino acid	*Actin*			*Myosin A*		
	Rabbit[25] *skeletal muscle*	*Sheep*[12] *uterus*	*Horse leucocytes*	*Horse*[16] *skeletal muscle*	*Horse*[16] *esophagus*	*Horse leucocytes*
Lys	45.0	44.5	44±4	90	76	79±1
His	17.1	17.5	17±1	15	12	10±2
Arg	41.8	43.3	44±2	46	43	39±1
Asp	80.5	77.3	86±1	80	103	74±8
Thr	67.6	61.1	71±4	36	38	34±2
Ser	57.1	62.8	57±0	36	33	25±4
Glu	93.5	93.1	128±9	172	178	133±10
Pro	43.8	43.1	60±0	22	17	1±0
Gly	65.8	66.3	67±1	37	28	32±1
Ala	70.1	69.8	73±3	75	77	54±12
S-CM-Cys	11.0	12.1	–	8.2	8.4	–
Cys	–	–	11±1	–	–	–
Val	43.6	43.0	40±0	40	38	30±5
Met	29.0	27.1	28±0	21	21	13±3
Ile	64.5	62.6	56±1	34	33	21±4
Leu	59.6	64.3	70±2	79	99	62±7
Tyr	33.8	32.6	31±0	14	14	11±3
Phe	27.1	28.1	30±0	29	27	19±4

Electron micrographs showed filamentous structures with a helical form, a diameter of about 7.5 nm and a pitch of 35.0 nm as shown in Fig. 3. These structures in F-actin of leucocytes were indistinguishable from those of skeletal muscle or arterial smooth muscle F-actin[13,14]. The above results show no significant difference between actins of leucocytes and of muscle.

Nature of myosin A from leucocytes

The ATPase of leucocytic myosin A was activated by Ca^{2+} and EDTA and inhibited by Mg^{2+} at high ionic strength, like that of myosin A from skeletal[15] or arterial muscle[13], (left side of Table II). The Ca^{2+}-dependent ATPase activity was enhanced at high rather than low ionic strength, unlike skeletal myosin A ATPase. Ca^{2+}-dependent ATPase of myosin A from bovine carotid arterial and equine esophageal smooth muscle had similar character to that from leucocytes[13,16], as

TABLE II

ATPase ACTIVITY OF MYOSIN AND SYNTHETIC ACTOMYOSIN FROM LEUCOCYTES AT DIFFERENT KCl CONCENTRATIONS

ATPase activity was measured in 10 mM Tris–maleate buffer (pH 7.0) and 1 mM ATP with or without 10 mM $MgCl_2$, $CaCl_2$ or EDTA at 25 °C. The reaction time was 10 min. Protein concentration: actin 0.21 mg/ml, myosin 0.30 mg/ml.

	P_i liberated (μmoles/min per mg myosin protein)			
	Myosin A		*Mixture of myosin A and actin*	
	0.55 M KCl	*0.06 M KCl*	*0.55 M KCl*	*0.06 M KCl*
No metal ion	0.034	0.044	0.012	0.019
$MgCl_2$ (10 mM)	0.012	0.008	0.008	0.019
$CaCl_2$ (10 mM)	0.067	0.015	0.026	0.026
EDTA (1 mM)	0.072	0.044	0.022	0.010

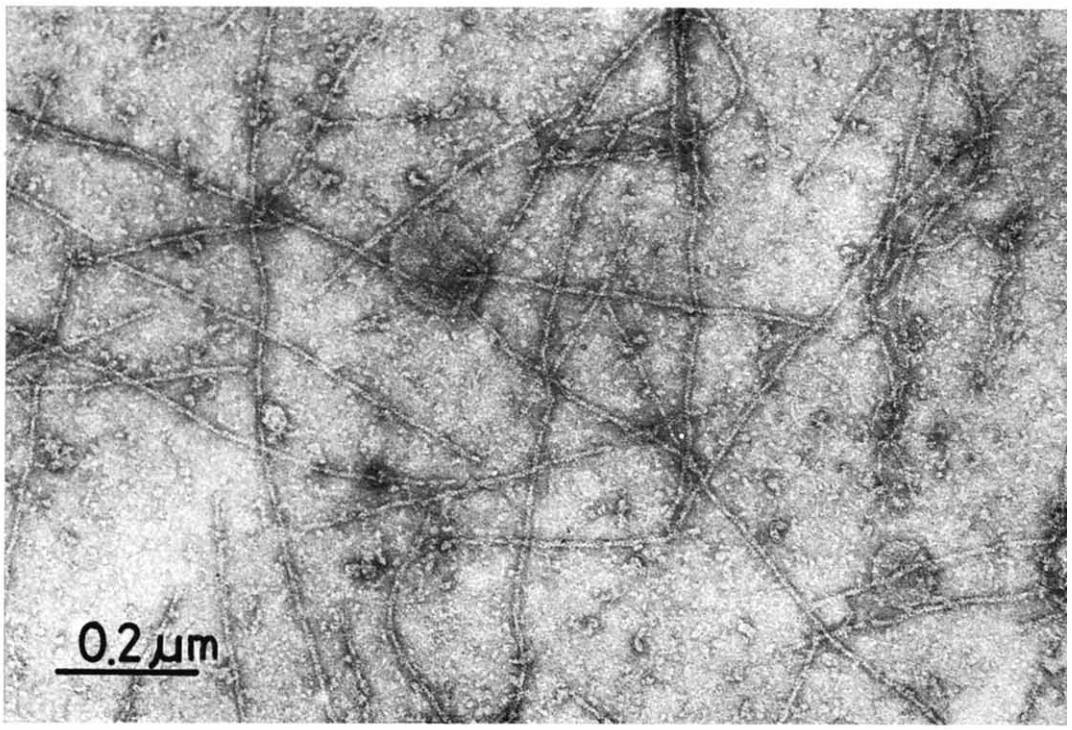


Fig. 3. Electron micrograph of leucocyte actin. The mixture contained 0.1 M KCl, 50 mM Tris–maleate buffer (pH 7.0) and 0.3 mg of protein per ml.

regards activity at low or high ionic strength, suggesting that myosin A from leucocytes resembled that of smooth muscle.

On analytical ultracentrifugation the protein gave a single peak, with an $s_{20,w}$ value of about 6.6 S (Fig. 4). This value showed no major differences with 6.43 S, 6.16 S and 5.9 S of the sedimentation coefficients of myosin A from skeletal muscle[17], canine cardiac muscle[18] and equine esophageal smooth muscle[16], respectively.

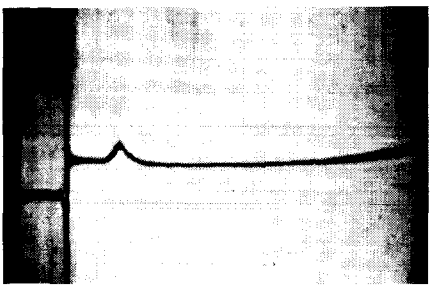


Fig. 4. Sedimentation pattern of leucocyte myosin. The mixture contained 1.5 mg/ml protein, 0.6 M KCl and 10 mM Tris–maleate buffer (pH 7.0). Ultracentrifugation was carried out at 51200 rev./min at 24 °C at an angle of 70°. The photo was taken 32 min after reaching maximal speed. $s_{20,w}$, 3.2 S.

In the amino acid composition of the leucocytic myosin A, the molar ratios of glutamic acid, alanine, methionine and isoleucine in the leucocytic myosin A were about five-sixth of those of skeletal muscle[25], as far as calculations using the mean values are valid. The amino acid composition of the leucocytic myosin A seemed also to have no major differences from that of equine esophageal smooth muscle[16], except aspartic acid and proline.

At low ionic strength skeletal myosin A is known to polymerize, forming aggregates. Electron micrographs of these show thick filaments with lateral projections and a diameter of about 15.0 nm. Electron micrographs of the leucocytic myosin A were taken under similar conditions to those used for skeletal muscle myosin A. These showed filaments with characteristic fine projections at the both ends, like those of aggregates of skeletal muscle myosin A. However, unlike the latter, the filaments did not taper at the ends (as shown in Fig. 5). The filaments were about 0.3 μm long and about 15.0 nm wide. They were a little shorter than those of cardiac muscle myosin aggregate (0.7 μm, ref. 13), but about equal in length to those of myosin A from equine esophageal smooth muscle[16], or bovine arterial smooth muscle[13]. The filaments appeared very similar to the thick filaments of actomyosin. These formed when leucocytic actomyosin was dissolved in solution of low ionic strength containing rather high concentrations of ATP and Mg^{2+}, under conditions giving the so-called clearing response of skeletal muscle actomyosin.

As above described, the leucocytic myosin A seems to be closer to myosin A from smooth muscle rather than to that from skeletal muscle, taking into account such characteristics as its ATPase activities and the electron microscopic appearance of aggregates.

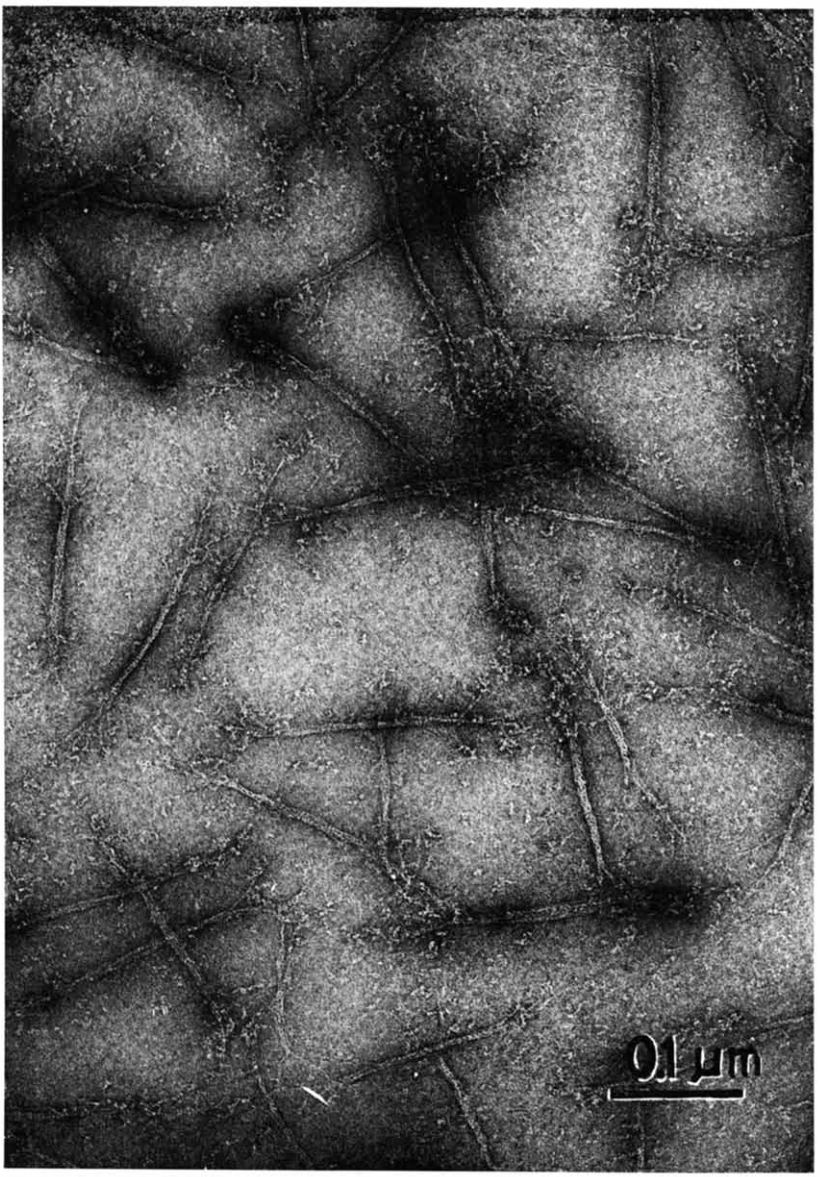


Fig. 5. Electron micrograph of leucocyte myosin. The mixture contained 0.06 M KCl, 10 mM Tris–maleate buffer (pH 7.0) and 0.3 mg/ml protein.

Synthetic actomyosin of leucocytes

As suggested in a previous paper[2,3], the contractile protein of leucocytes may be a complex of myosin A and actin. This possibility was studied by testing whether a mixture of myosin A and actin, which had been prepared separately, showed the characteristic features of synthetic actomyosin in the presence of ATP and Mg^{2+}.

(*a*) *Superprecipitation.* As shown in Fig. 6 the turbidity of myosin A in a solution at low ionic strength increased on addition of actin, demonstrating typical superprecipitation. The superprecipitation was not affected by addition of EGTA, and was not sensitive to Ca^{2+}, as observed with leucocytic actomyosin, probably due to extraction of actin at 0 °C from an acetone powder of actomyosin, not of natural actomyosin.

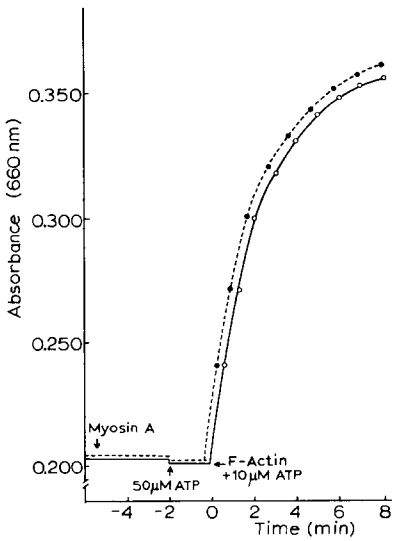


Fig. 6. Superprecipitation of synthetic actomyosin constituted from actin and myosin. The mixture contained 0.06 M KCl, 5 mM $MgCl_2$, 10 mM Tris–maleate buffer (pH 7.0) with 0.1 mM $CaCl_2$ (○————○) or 1 mM EGTA (●-------●) in 3.0 ml. The protein concentrations were 0.21 mg of leucocyte actin per ml and 0.3 mg of leucocyte myosin per ml.

(*b*) *ATPase activity.* The Mg^{2+}-dependent ATPase activity of the leucocytic myosin A was stimulated about 2.4-fold on addition of the leucocytic F-actin at low ionic strength as shown in Table II. This shows that the enzyme has actomyosin-type ATPase activity, the leucocytic myosin A reacting with added F-actin. The activation was much less than that observed with myosin A and F-actin, both from skeletal muscle, and was similar to that observed with a mixture of cardiac myosin A and skeletal muscle actin[21] or a mixture of myosin A and actin from arterial smooth muscle[13].

(*c*) *Change in viscosity on addition of ATP.* When a mixture of leucocytic actin and myosin A was adjusted to an ionic strength of 0.6, the viscosity of the mixture increased markedly, indicating the formation of synthetic actomyosin. Addition of ATP and Mg^{2+} to this mixture caused a rapid decrease in viscosity. This phenomenon is explained as due to dissociation of the synthetic actomyosin into myosin A and actin, as observed in the case of skeletal muscle actomyosin. The sudden decrease in viscosity was followed by a gradual increase in viscosity towards the initial level, as hydrolysis of ATP proceeded with time.

The results listed in (a) to (c) above indicate that the proteins isolated from

leucocytes correspond to myosin A and actin, respectively, and, furthermore, that these two proteins combine to form synthetic actomyosin.

Interaction between myosin A and F-actin from skeletal muscle or leucocytes

A mixture of the leucocytic myosin A and skeletal muscle F-actin in a medium at low ionic strength demonstrated superprecipitation in the presence of ATP and Mg^{2+}, as observed with synthetic actomyosin preparations from skeletal muscle and leucocytes (Fig. 7A). A mixture of skeletal muscle myosin A and the leucocytic F-actin also showed superprecipitation by an addition of ATP in the presence of Mg^{2+}.

Superprecipitation of actomyosin synthetized from skeletal muscle F-actin and leucocytic or skeletal muscle myosin A was investigated at high (20 mM) and low (0.5 mM) concentration of $MgCl_2$. As shown in Fig. 7A, actomyosin synthetized from leucocytic myosin A demonstrated strong and rapid superprecipitation at high Mg^{2+} concentration but rather weak superprecipitation at low Mg^{2+} concentration. On the contrary, actomyosin synthetized from skeletal muscle myosin A showed marked superprecipitation at low Mg^{2+} concentration, but no significant superprecipitation at 20 mM $MgCl_2$ (Fig. 7B). The effect of the Mg^{2+} concentration on the superprecipitation of synthetic actomyosin composed of leucocytic myosin A and the skeletal muscle F-actin was very similar to that of the leucocytic contractile protein, as reported previously[2,3], and to that of carotid artery[13].

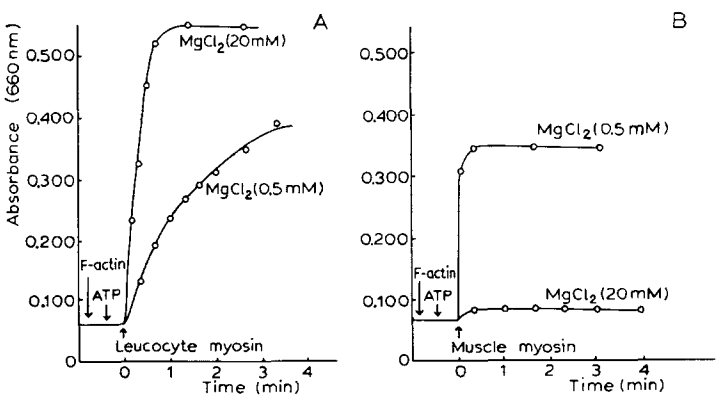


Fig. 7. Effect of Mg^{2+} on superprecipitation of synthetic actomyosin composed of (A) muscular actin and leucocyte myosin A or (B) muscule myosin A. The reaction mixture contained 0.06 M KCl, 0.1 mM $CaCl_2$, 0.01 mM ATP, 10 mM Tris–maleate buffer (pH 7.0) and $MgCl_2$ at the concentration in each figure in 3.0 ml at 25 °C. Protein concentration, (A) 0.28 mg skeletal muscle F-actin per ml and 0.32 mg leucocyte myosin per ml, (B) 0.28 mg skeletal muscle F-actin per ml and 0.25 mg skeletal muscle myosin per ml.

These results indicate that leucocytic actin and myosin A can combine and that the resulting synthetic actomyosin has contractile activity. The results also suggest that leucocytic myosin A resembles myosin A from smooth muscle more closely than that from skeletal muscle with respect to the optimal concentration of Mg^{2+} necessary for the interaction of myosin A and F-actin.

DISCUSSION

This paper shows that myosin A and actin isolated separately from leucocytes can combine to form synthetic actomyosin. The contractile protein we isolated from leucocytes was fundamentally similar to that from muscle, and the main components of the latter are myosin A and actin.

One problem in isolation of actin from leucocytes is that of obtaining sufficient starting material. Only 300 to 500 mg dry weight of acetone powder of actomyosin were obtained from 20 l of equine whole blood. Thus, to obtain sufficient material for extraction of actin (usually 3 to 5 g dry weight), several batches of acetone powder were made, stored in a deep freezer, and later pooled. Another difficulty is to prevent denaturation of actin. However, taking precautions to prevent this, we succeeded in isolating actin from leucocytes.

The only other free cells from which actin has been isolated are Plasmodium[22] and platelets[23]. Our preparations of leucocytic actin is similar in nature to those from skeletal muscle, carotid artery[13] and Plasmodium[22], suggesting that actins from various sources have similar characteristics.

The leucocytic actomyosin isolated was more like that of arterial smooth muscle than that of skeletal muscle, as regards the optimal concentration of Mg^{2+} necessary for its superprecipitation[13]. Accordingly, for isolation of myosin A from leucocytes we used the method employed for isolation of that of smooth muscle[13]. Leucocytic myosin A precipitated with 45 to 55% saturation of $(NH_4)_2SO_4$. On the other hand, a crude preparation of skeletal myosin A precipitated with 40 to 50% saturation of $(NH_4)_2SO_4$. Using these characteristics of the solubility of myosin A at various pH values and its precipitation with $(NH_4)_2SO_4$, we succeeded in preparing myosin A from natural actomyosin of leucocytes.

The sedimentation coefficient of leucocytic myosin A and its reactivity with actin were similar to those of skeletal muscle myosin A, but the dependence of the ATPase activity on Mg^{2+} and Ca^{2+} and the dependence of superprecipitation on Mg^{2+} were similar to those of myosin A from arterial smooth muscle. Namely, the optimal concentration of Mg^{2+} necessary for the interaction of the leucocytic myosin A with actin was similar to that of myosin A with actin from smooth muscle, and much higher than that of myosin A with actin from skeletal muscle.

Electron micrographs showed that the aggregates of leucocytic myosin A were shorter than those of skeletal myosin A, but rather like those of myosin A from smooth muscle [13,16], which are fan-shaped at both ends. These difference may be related to differences in behavior or motility of skeletal and smooth muscle. But whatever the reason, it seems that the contractile protein of leucocytes is similar to that of smooth muscle.

Recently Booyse *et al.*[24] reported that the effects of Ca^{2+} and EDTA on the ATPase activity of myosin A from thrombocytes were different from those on the ATPase activity of myosin A from striated muscle.

The ATPase of the leucocytic myosin A was stimulated by a factor of 2.4 on addition of the leucocytic F-actin. This value was only one twentieth of that observed when both myosin A and F-actin were from skeletal muscle, but stimulation of myosin A ATPase from smooth muscle on addition of F-actin from skeletal or

smooth muscle was to almost the same degree as the case of leucocytic myosin A on addition of F-actin[13,16].

These facts indicate that the leucocytic actomyosin is, physicochemically, rather more close to smooth muscle than to skeletal muscle actomyosin. This situation seems to be attributed to the differences in myosin A rather than actin.

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