Identification of Myosin A, Actin and Native Tropomyosin Constituting of Arterial Contractile Protein (Myosin B) and Their Characteristics

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The artery may be understood as an organ involved in blood circulation through contraction and relaxation. Therefore, in considering "metabolism" performed in the arterial wall, it is very important even in clinical view to clarify the action mechanism or the characteristic features of the contractile protein localized in arterial smooth muscle which is considered to be the essential substance for contraction and relaxation of the artery.

From the above standpoint, in 1967 we attempted to extract natural actomyosin, that is myosin B being a Ca\textsuperscript{2+} sensitive contractile protein, from arterial wall\textsuperscript{1,2}

In this paper, it is clarified that the contractile protein from arterial wall is a protein complex mainly consisting of myosin A and actin as an elementary contractile protein and of the so-called native tropomyosin as a contractile regulatory protein, in the presence of which Ca\textsuperscript{2+} affects the interaction of myosin A and actin, as previously established in myosin B of skeletal muscle\textsuperscript{3}. Furthermore, it will be presented that a thin filament observed electronmicrographically in both arterial and capillary walls was identified as the filament containing actin through the characteristic arrowhead structure formed with heavy-meromyosins prepared from skeletal myosin A and arterial myosin A.

**MATERIAL AND METHOD**

1) Myosin B (natural actomyosin)

Arterial myosin B was prepared from bovine carotid arteries by a modification of the method developed by Rüegg and Schirmer\textsuperscript{4} as described previously by us\textsuperscript{1,2}. The fresh arteries were, at first, washed well with ice cold 0.15M saline

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**Key Words:** Arterial Contractile Protein, Natural Actomyosin (Myosin B), Myosin A, Actin, Native Tropomyosin, Troponin, Tropomyosin, ATPase, Arrowhead Structure

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Carotid minced muscle
+ 5 vol. Guba-Straub's sol.
homogenate (5 min)
| passing through gauze
washing with H₂O
| extraction with 5 vol. 0.2 mM NaHCO₃
| for 16 hr (at 4°C)
| filtration through gauze
| centrifuge 4 × 10⁴ g, for 30 min
| sed.
| sup.
| fractionation with (NH₄)₂SO₄
| 37% ppt.
| 65% ppt.
| dialysis against
| 0.2 mM NaHCO₃
| centrifuge 1 × 10⁴ g, 20 min
| sup. (native tropomyosin)

Fig.2. Preparation method of arterial native tropomyosin

solution and after removing adventitia the arteries were immersed in 50% glycerol for 24—48 hr at −18°C. The glycerinated arteries were well washed with ice cold redistilled water and cut in small pieces after blotting with gauze. The pieces of arteries were homogenized for 3 min at 0°C in warring blender with 5 volumes of the extracting solution consisting of 20 mM histidine-buffer (pH 7.0), 0.05 M KCl and 1 mM ATP. The homogenate was stirred with magnetic stirrer for 2 hr at 4°C and then centrifuged at 4×10⁴ g for 1 hr. The resultant supernate was dialyzed at 4°C against 5 mM histidine buffer (pH 7.0) containing 0.05 M KCl to obtain the soluble contractile protein as flocculent precipitate through removing ATP. The resultant precipitate was dissolved in 0.6 M KCl followed by centrifugation at 1×10⁴ g for 15 min. The supernate was again dialyzed against 5 mM histidine buffer (pH 7.0) containing 0.24 M KCl at 4°C. The resultant precipitate was again dissolved in 0.6 M KCl. The insoluble material was removed by centrifugation at 1×10⁴ g for 10 min as sediment. The soluble supernate was used as the arterial myosin B. Its yield was approximately 7 mg of protein per 1 g (wet weight) of arterial wall. The contamination of microsomal vesicles is thought to be negligible, for the Mg²⁺-dependent ATP-ase activity per unit protein is much lower by a factor of 10⁻² than microsomal vesicles.

2) Myosin A:

Arterial myosin A was firstly extracted from arterial myosin B by the method already reported by us (Fig. 1)⁵ and later, by a modification of the procedure developed by Yamaguchi in the preparation from esophagus.⁶ That is, in the first step, ATP, MgCl₂, EGTA and DTT were added to the solution containing arterial myosin B dissolved in 0.6 M KCl so as to adjust their each final concentration to 10 mM, 10 mM, 10mM and 2 mM, respectively. Then, the pH of the mixture was adjusted to 7.8 with KOH. This solution in which myosin B dissociates into myosin A and F-action was centrifuged at 10×10⁴ g, for 2.5 hr to remove F-action as the sediment. The upper half layer of the supernate was used for preparation of myosin A. The ionic strength of the supernate was reduced to 0.06 by addition of 9 fold of water and the pH was adjusted to 5.3 with 1N HCl to obtain myosin A as isoelectric precipitates. In the last step, the precipitates were dissolved in 0.6 M KCl (pH7.6). This was used as the preparation of myosin A.

In modified method, the supernate of centrifuged myosin B was used for the preparation. The supernate was fractionated with saturated ammonium sulfate, after the supernate was dialyzed against 0.5 M KCl (pH7.0) for omitting ATP, MgCl₂ and EGTA. Arterial myosin A was obtained as the precipitates in 45—55% fraction of ammonium sulfate. The precipitates were dissolved through dialysis against 0.5 M KCl with 2 mM DTT (pH7.0). There was no appreciable difference in properties between myosin A obtained by the former method and by the above described different method, but the yield of myosin A was better by the latter than by the former.

Cardiac or skeletal myosin A was prepared form bovine cardiac muscle (left-ventricle) or from back skeletal muscle of rabbit by the method employed by Perry⁷ with slight modifications.

3) Actin:

Actin from arterial wall was extracted from aceton powder of fresh bovine carotid arteries. The aceton powder was prepared by the method described by Katz et al. for preparation of cardiac actin⁸

At first, crude G-actin was extracted from the arterial aceton powder with 5 volumes of 0.2 mM

*Japanese Circulation Journal* Vol. 37, March 1973
ATP-ascorbic acid solution (pH 7.5) at 0°C (cold actin) or at 25°C (warm actin) and after removing insoluble materials by centrifugation at 3.5 x 10^8 xg for 1 hr, G-actin solution was stirred for 2 hr in the presence of both 0.1 M KCl and 2 mM MgCl₂ to polymerize it to F-actin at room temperature.

- The mixture was centrifuged at 10 x 10^8 xg for 2.5 hr to obtain the F-actin as a sediment. The sediment was dissolved in a small amount of 0.05 M KCl. The solution was again centrifuged at 2 x 10^8 xg for 30 min and the supernatant was used as a purified F-actin preparation.

Fig. 3. Effects of Ca²⁺ on superprecipitation at 20°C of arterial myosin B. Protein concentration, 0.45 mg/ml; ionic strength, 0.06; Tris-maleate buffer, 10 mM (pH 7.0); ATP, 1.0 mM; MgCl₂, 10 mM total vol. 3.0 ml. The reaction was started by the addition of ATP. Free Ca²⁺ concentration was set up by Ca-EGTA buffer, EGTA concentration being 3.3 mM. Concentrations of Ca²⁺ in the reaction medium were 1 x 10^{-6} M (□□□□), 1 x 10^{-6} M (x-x), 5 x 10^{-6} M (△-△), 1 x 10^{-5} M (□-□), 1 x 10^{-7} M (○-○) and nearly zero obtained with only EGTA without added CaCl₂ (▲-▲). At the points designated as □□□□ and □□□□, electronmicrograph of arterial myosin B was taken as shown in Fig. 5, 6 and 7.

Fig. 4. Effects of added Mg²⁺ on superprecipitation at 20°C of arterial myosin B. Protein concentration, 0.45 mg/ml; ionic strength, 0.06; Tris-maleate buffer, 10 mM (pH 7.0); ATP, 0.5 mM; Ca²⁺ concentration, 1 x 10^{-6} M (Ca-EGTA buffer); total vol., 3.0 ml. The reaction was started by the addition of ATP. Final concentration of added MgCl₂ was 20 and 10 mM (□□□□), 5 mM (○○○○), 1 mM (△-△), 0.5 mM (□□□□). ——, with 3 mM EDTA.
Cardiac and skeletal actin was prepared from canine cardiac muscle (left-ventricle) and back muscle of rabbit at 0°C by the method employed by Katz.  

4) Native-tropomyosin:

Arterial native-tropomyosin (TMN) was prepared from bovine carotid arteries by a modification of the method described by Ebashi for preparation of native-tropomyosin from striated muscle. The outline is shown in Fig. 2. That is, fresh arteries stripped from adventitia was minced and homogenized with 5 vol. of Guba-Straub's solution. The homogenate was squeezed by gauze and the material left on gauze was washed with water, then was added to it 5 vol. of 0.2 mM NaHCO₃ and the mixture was stirred gently with magnetic stirrer for about 16 hr at 4°C. It was filtered through gauze, then the filtrate was centrifuged at 4x10⁶xg for 30 min. To the resultant filtrate was added solid ammonium sulfate so as to make its final concentration 37%, then the resultant precipitate was removed through centrifugation at 5000xg for 10 min. Then, ammonium sulfate was further added to he supernate until its concentration attained to 65%. The resultant precipitate was dialyzed against 0.2 mM NaHCO₃ solution, then it was centrifuged at 1x10⁶xg for 20 min and the resultant supernate was used as arterial native-tropomyosin (TMN) in the experiments.

5) Heavy meromyosin (H-meromyosin):

Arterial and skeletal H-meromyosins were prepared by a modification of the method described by Scem-Györgyi. Thus, H-meromyosins were obtained in the precipitates from 45% to 60% saturated ammonium sulfate fraction, after the protein solution was digested with trypsin 15°C for 10 min. The both H-meromyosins were stored in a solution containing 0.05 M KCl and 0.5 mM EDTA (pH7.0) at a concentration of about 15 mg of protein per ml.

6) Preparation of synthetic actomyosin:

Actomyosin was prepared from myosin A and F-actin of various source as described in the legend of the related figure.

7) Measurement of superprecipitation:

The superprecipitation was measured by the turbidometric method of Ebashi using a Hitachi UV-VIS spectrophotometer.

8) ATP-ase activity:

Japanese Circulation Journal  Vol. 37, March 1973
The ATP-ase activity of synthetic actomyosin or acto-H-meromyosin was determined in the coupled system with pyruvate kinase (Py-k) and phosphoenol pyruvate (PEP) as an ATP generating system by measuring the time dependence of pyruvate liberation according to the method of Reynolds et al.\textsuperscript{11} The concentrations of Py-k and PEP used were so high that the rate of the overall reaction was independent of the rate of the kinase reaction. The reaction was started by an addition of ATP. At appropriate times, 0.5 ml of the reaction mixture was transferred to a solution containing 0.2 ml of 2.5 mM 2,4-dinitrophenyldiazin in 3 M HCl to stop the reaction. This coupled system was not influenced by the Mg\textsuperscript{2+} concentration in the medium over the range from 0.5 to 20 mM.

In some experiments, the ATP-ase activity was measured by determining the amount of Pi liberated from ATP by the method of Marsh.\textsuperscript{12}

9) Ultracentrifuge:

A Hitachi Model UCA-1 analytical ultracentrifuge was employed for the sedimentation-velocity measurement.

10) Amino acid analysis:

Amino acid analysis was conducted by the use of a Hitachi KLA-3B amino acid analyser.

11) Viscosimetric determination:

This was performed in an Ostwald viscosimeter with 2 ml capacity at 25°C.

12) Electron micrographs:

Micrographs of the proteins were taken by the negative staining technique of Huxley.\textsuperscript{13} One drop of an ice-cold preparation of the protein was placed on a microgrid coated with collodion carbon. The preparation was negatively stained with 1% uranyl acetate, and examined in a Nihon Denki Model JEM 100B electron microscope with an acceleration voltage of 80 KV. Electron micrographs of arterial F-actin reacted with H-meromyosins were taken by the following procedure. That is, drops of the F-actin solutions were applied to microgrids coated with collodion carbon. After removal of the F-actin solution, a drop of H-meromyosins was added to the grids and allowed to stay for 1–3 min before removal with filter paper. The grids were then stained with 1% uranyl acetate.

In the case of taking electronmicrographs of arterial wall or kidney, arteries trimmed off...
adventitia or thin sectioned pieces of kidney were treated with 50% cold glycerol for 1 hr along a modification of the method of Ishikawa et al. The glycerinated tissues were washed with the standard solution consisting of 50 mM KCl, 5 mM MgCl₂, 10 mM Tris-HCl buffer (pH 7.4) for 12 hr, then they were fixed by 2.5% glutaraldehyde-cacodylate buffer (pH 7.4) with 2 mM CaCl₂ either directly or after the reaction with H-meromyosin for 12 hr; postfixation was carried out in 1% osmium tetroxide. The fixed tissues were dehydrated with graded concentrations of ethanol containing uranyl acetate. They were embedded in Epon and stained with both uranyl acetate and lead citrat.

13) Protein determination:
The amount of protein was determined by the method of Lowry et al. using bovine serum albumin as standard.

14) Chemicals:
All chemicals used were analytical grade. Various free Ca²⁺ concentrations were set up by means of Ca-EGTA buffer (at pH 7.0) prepared in the manner described by Portzehl et al. The water used was distilled in a glass vessels.

RESULTS
(A) Arterial myosin B (natural actomyosin)
It is a well-known property of myosin B, the natural contractile protein from striated muscle, to undergo superprecipitation, being considered as an in vitro model of muscular contraction, at low ionic strength in the presence of not only Mg\textsuperscript{2+} and ATP but also Ca\textsuperscript{2+}.10

As shown in Fig. 3, the arterial myosin B extracted by us showed a marked superprecipitation on addition of small amount of ATP and Ca\textsuperscript{2+} in the presence of large amount of MgCl\textsubscript{2}. And, it was evident that the degree of superprecipitation which was measured by an increase in absorbance at 660 μm was associated with the concentration of free Ca\textsuperscript{2+} in the medium. That is, a superprecipitation occurred significantly at concentrations above 1x10\textsuperscript{-6} M Ca\textsuperscript{2+} and increased up to 1x10\textsuperscript{-4} M Ca\textsuperscript{2+}, whereas the elimination of Ca\textsuperscript{2+} in a medium containing 1.0 mM EGTA resulted in a clearing response corresponding to muscular relaxation and in a complete inhibition of superprecipitation.

Fig. 4 shows the effect of Mg\textsuperscript{2+} concentration in the medium on the superprecipitation of the arterial myosin B. Up to 20 mM MgCl\textsubscript{2} added, the superprecipitation was enhanced. In a medium containing 5 mM EDTA, 0.1 mM ATP and 1x10\textsuperscript{-4} M Ca\textsuperscript{2+} but no added Mg\textsuperscript{2+} the protein showed no superprecipitation.
Effect of ATP concentration

\[(\text{Pi \, \mu moles/mg/min.})^{-1}\]

\[1/([\text{mM}] \text{ATP})\]

**Fig. 9.** Lineweaver-Burk plot of arterial myosin B ATP-ase.
Protein concentration, 0.45 mg/ml; ionic strength, 0.06; Tris-maleate buffer, 10 mM (pH 7.0); MgCl₂, 10 mM; Ca²⁺; 1 \times 10⁻⁴ M (3.3 mM EGTA-Ca buffer).
Total vol., 3.0 ml. Temperature, 20°C. Reaction time, 2 min.

**Fig. 10.** Electronmicrograph of arterial myosin A aggregates.
Arterial myosin A dissolved in 0.6 M KCl was added to 10 mM Tris-maleate buffer (pH 7.0). The final KCl concentration in this preparation was 0.10 M.
From the above results, it was evident that the arterial myosin B required not only Mg$^{2+}$ and ATP but also Ca$^{2+}$ for superprecipitation as well as that from striated muscle. However, the optimal concentration of Mg$^{2+}$ for superprecipitation was very high in arterial myosin B as compared with that (40 μM) in skeletal myosin B$^{29}$ This is considered as one of the characteristic features of arterial myosin B.

In order to observe the ultrastructure of arterial myosin B in the clearing response and in the superprecipitation, electronmicrographs were taken of the reaction mixture containing EGTA alone instead of Ca-EGTA buffer just after an addition of ATP (© on the curve 6 in Fig. 3) and, also, of the reaction mixture with 10$^{-5}$ M Ca$^{2+}$ after the achievement of superprecipitation (© on the curve 1 in Fig. 3).

In clearing response (a), thick and thin filaments of 150Å and 80Å in diameter were visible separately (Fig. 5). In this micrograph, it was found that the thick filaments possessed the fine projections characteristic of myosin aggregates from skeletal muscle$^{13}$ at their both ends.
Fig. 12. Viscosity change of cardiac and arterial G-actin after an addition of either KCl or KCl and MgCl₂ at 25°C.

- a) Cardiac G-actin; protein concentration, 2.7 mg/ml. At 0 min, KCl was added to the G-actin solution so as to get 0.1 M in the final concentration.
- b) Arterial G-actin; protein concentration, 0.44 mg/ml. At 0 min, KCl and MgCl₂ were added to the G-actin solution so as to get 0.1 M and 2 mM in the final concentration, respectively.

and the thin filaments possessed helical structure with pitch of about 350Å characteristic of F-actin from skeletal muscle.³ In superprecipitation (b), the formation of large aggregates was found (Fig. 6). In the preparation (c) prior to an addition of ATP, it was found that thick and thin filaments were arranged parallel each other without formation of large aggregates (Fig. 7).

From these results, it is suggested that thick filaments correspond to myosin aggregates and thin to F-actin filament and that both filaments dissociated each other in clearing response and joined together to form large aggregates in superprecipitation. It is considered that contraction and relaxation of arterial wall are performed through the joining together and the dissociation of the myosin filament and F-actin filament, respectively. As shown in Fig. 8, the degree of superprecipitation of arterial myosin B was closely associated with its ATP-ase activity. The free Ca²⁺ and MgCl₂ concentrations at which the ATP-ase activity of myosin B was half activated were about 1x10⁻⁶ M and 3 mM, respectively. The ATP-ase reaction of arterial myosin B conformed to Lineweaver-Burk equation within the ATP concentration of 0.1 mM - 1 mM in the reaction medium containing 0.06 M KCl, 10 mM MgCl₂, 10⁻⁴ M Ca²⁺ and 10 mM Tris-mal. buffer (pH7.0) (Fig. 9). The Km and Vmax were 0.212 mM and 0.096 μmoles Pi/mg protein/min.

(B) Myosin A and actin of arterial wall

Since it was suggested that arterial contractile protein consisted of myosin A and actin as well as that from skeletal muscle, it was attempted to extract separately myosin A and actin from arterial tissue. And, it was investigated whether or not they had any characteristic feature different from myosin A or actin of other organs and, further, whether or not the mixture of myosin A and actin might play as the actomyosin.

Arterial myosin A possesses ATP-ase activity which is activated by Ca²⁺ (0.0185 μmoles Pi/mg protein/min at 5.0 mM CaCl₂) and inhibited by Mg²⁺ (0.0040 μmoles Pi/mg protein/min at 10 mM MgCl₂) in high ionic strength. Mg²⁺ and Ca²⁺ showed the similar effects on ATP-ase activity of cardiac myosin A (0.0065 and 0.0177 μmoles Pi/mg protein/min at 10 mM MgCl₂ and at 5.0 mM CaCl₂, respectively).

In skeletal myosin A, it has been found that myosin monomer turns to aggregated form at low ionic strength and the myosin aggregates possess the characteristic lateral projections in electronmicroscopic structures.¹³ Electronmicrograph of arterial myosin A at low ionic strength (Fig. 10) revealed filamentous structure similar to thick filaments shown in Fig. 5. The arterial myosin aggregates possessed fine projections characteristic of skeletal myosin aggregates at both ends, the shape was rod without tapering and their width was about 150Å. Their length was about 0.3μ, which was shorter than that of cardiac myosin aggregates shown in Fig. 11.

As shown in Fig. 11, cardiac myosin aggregates exhibited longer (about 0.5 μ) but the same tapering filamentous structure possessing lateral projections as that reported in skeletal myosin.
in the presence of MgCl₂ and KCl as clarified in G-actin from striated muscle. However, as shown in Fig. 12, the speed of polymerization was slow in arterial G-actin compared with cardiac G-actin. And depolymerization of arterial F-actin to G-actin was impossible, being different from F-actin of striated muscle.

Arterial F-actin from cold actin showed electronmicrographically the same filamentous structure of about 80Å in diameter and helical structure with pitch of about 350Å as thin filaments shown in Fig. 5 (Fig. 13). This structure of arterial F-actin is very similar to that reported in cardiac and skeletal F-actin¹³. Electronmicrographs of arterial F-actin reacted with skeletal H-meromyosin or arterial H-meromyosin showed the characteristic arrowhead structure as shown in Fig. 14 and 15, respectively. This arrowhead structure is considered to be formed by attachment with uniform direction of H-meromyosin to F-actin and this structure has been generally accepted to be characteristic features of the reaction between F-actin and H-meromyosin¹³.

Amino acid analysis of arterial actin and myosin A was compared with that of skeletal, and cardiac myosin A and actin (Table I), finding that amino acid compositions of actins from various organs were very similar. On the other hand, in myosin A, contents of lysine, threonine, and glutamic acid were different from those of myosin A from striated muscle.

In the following experiment, it was investigated whether or not the mixture of arterial myosin A and F-actin brought about the superprecipitation by an addition of ATP in the low ionic strength solution containing Mg²⁺.

As shown in Fig. 16, the right tube containing myosin A alone and the left tube containing F-actin alone did not show any change even after an addition of ATP, whereas the middle tube containing both myosin A and F-actin made a superprecipitated mass about 15 min after an addition of ATP.

From the above findings, it is concluded that the contraction of arterial smooth muscle without striated structure is performed through the interaction between myosin A and F-actin in the presence of ATP and Mg²⁺ as in the case of striated muscle, although arterial myosin A seems

Fig. 13. Electronmicrograph of arterial F-actin.
Protein concentration, 0.1 mg/ml. The pitches of the helical structure were shown by (+).
Fig. 14. Electronmicrograph of arterial F-actin reacted with H-meromyosin from skeletal muscle.

Fig. 15. Electronmicrograph of arterial F-actin reacted with arterial H-meromyosin.

*Japanese Circulation Journal* Vol. 37, March 1973
to be somewhat different from that of striated muscle in ultrastructure of myosin aggregates and in amino acid composition.

(C) Arterial regulatory protein (native-tropomyosin)

Actomyosin synthesized from arterial myosin A and arterial F-actin from cold actin showed a very significant superprecipitation regardless of the presence of Ca\(^{2+}\)-chelating EGTA as shown in the right and left upper graphs of Fig. 17. However, actomyosin synthesized from arterial myosin A and arterial F-actin from warm actin did not show any superprecipitation in the medium containing Ca\(^{2+}\)-chelating EGTA as shown in the left lower graph of Fig. 17 and exhibited a very significant superprecipitation in the presence of Ca\(^{2+}\) as shown in the right lower graph of Fig. 17.

This findings may indicate that arterial F-actin from cold actin lacks in holding native-tropomyosin respondent to Ca\(^{2+}\) as clarified by Ebashi\(^3\) or Katz\(^28\) in F-actin from skeletal muscle. Accordingly, to obtain Ca\(^{2+}\)-sensitive F-actin, arterial cold actin was reacted with arterial native-tropomyosin at a ratio of 4:1 in protein amount in the presence of 2mM MgCl\(_2\) and 0.1M KCl for 2hr at 25\(^\circ\)C. Thus obtained F-actin was mixed with arterial myosin A to synthesize actomyosin. This actomyosin, as shown in the left middle graph of Fig. 17, showed only a very weak superprecipitation in the presence of Ca\(^{2+}\)-chelating EGTA, although it exhibited a very strong superprecipitation in the medium containing Ca\(^{2+}\) as shown in the right middle graph of Fig. 17. In this case, the addition of native-tropomyosin to F-actin polymerized in advance from G-actin extracted at 0\(^\circ\)C failed to get Ca\(^{2+}\)-sensitive F-actin.

In the experiment shown in Fig. 18, it was investigated how the F-actin from cold actin with or without native tropomyosin does act on manifestation of myosin A ATP-ase in the presence of Ca\(^{2+}\)
Fig. 16. Superprecipitation of the mixture of myosin A and F-actin from arterial wall. Right tube contains myosin A. Middle tube contains myosin A and F-actin. Left tube contains F-actin. The reaction mixture consisted of 10 mM MgCl₂, 10 mM Tris maleate buffer (pH 7.0), 0.06 M KCl and 0.5 mM ATP. Total vol., 3.0 ml. Myosin A concentration: 0.50 mg/ml. F-actin concentration: 0.50 mg/ml.

That is, it was found that myosin A ATP-ase activity was activated at either high or low Mg²⁺ concentration by F-actin from cold actin not combined with native-tropomyosin regardless of the presence of Ca²⁺-chelating EGTA, whereas it was significantly activated by F-actin combined with native-tropomyosin only in the presence of Ca²⁺ instead of EGTA.

From these results, it became evident that actomyosin synthesized from myosin A and F-actin containing native-tropomyosin behaved as myosin B that demonstrated Ca²⁺-sensitivity to the superprecipitation as well as to the ATP-ase activity.

Thus, it may be concluded that the contraction and relaxation of arterial wall is basically performed through the interaction of myosin A and F-actin associated with hydrolysis of ATP in the presence of Mg²⁺ and the interaction is regulated by Ca²⁺ concentration through native-tropomyosin contained in F-actin.

It was, further, investigated whether the arterial native-tropomyosin might have some effect on cardiac G-actin extracted at 0°C with regard to the Ca²⁺ sensitivity for superprecipitation. Actomyosin synthesized from cardiac myosin A and cardiac F-actin from G-actin extracted at 0°C demonstrated a significant superprecipitation regardless of the presence of Ca²⁺ as show in the left graph of Fig. 19, whereas cardiac actomyosin synthesized from F-actin combined with arterial native-tropomyosin could not show any superprecipitation in the presence of Ca²⁺-chelating EGTA.

*Japanese Circulation Journal* Vol. 37, March 1973
Fig. 17. Effect of Ca\textsuperscript{2+} and EGTA on the superprecipitation of arterial actomyosin composed of myosin A and either F-actin from cold actin with or without native tropomyosin or F-actin from warm actin.

At first, arterial myosin A was suspended in the reaction mixture containing 10 mM Tris-maleate buffer (pH 7.0), 10 mM MgCl\textsubscript{2}, 5 mM PEP, Py-k (10 \mu g) and either 1 \times 10^{-4} M Ca\textsuperscript{2+} (3 mM EGTA-Ca buffer) or 3 mM EGTA then 50 \mu g ATP was added to the reaction mixture. The ionic strength of the reaction mixture is 0.06. After ascertaining no change of turbidity in the reaction mixture, arterial F-actin of various types was added to the reaction mixture at 0 min to form arterial actomyosin and the reaction was started. Myosin A: 0.50 mg/ml. F-actin with or without TMN: 1.30 mg/ml. Total vol.: 3.0 ml. Temperature: 20°C. For details see text.
These results indicate that the arterial native tropomyosin can endow with Ca$^{2+}$-sensitivity to synthetic actomyosin from other organ.

(D) Interaction between myosin A or F-actin from arterial wall and F-actin or myosin A from cardiac muscle

In (A), it was found that arterial myosin B required much higher Mg$^{2+}$ concentration compared with skeletal myosin B for the superprecipitation.

Therefore, it was studied using arterial, cardiac and skeletal synthetic actomyosins how their superprecipitations and ATP-ase activities were influenced by low (0.5 mM) and high (10 mM) concentration of Mg$^{2+}$.

As shown in Fig. 20, in skeletal and cardiac actomyosins, the degree of the superprecipitation was higher at low concentration of Mg$^{2+}$ than at high Mg$^{2+}$ concentration, whereas, in arterial actomyosin, the superprecipitation was enhanced at high Mg$^{2+}$ concentration.

As shown in Fig. 21, the ATP-ase activity of arterial myosin A activated by arterial F-actin was augmented as increasing of Mg$^{2+}$ concentration (c-c) whereas, in cardiac and skeletal actomyosins (H-H and S-S), the ATP-ase activity of respective myosin A activated by respective F-actin was inhibited as increase in Mg$^{2+}$ concentration.

From these results, it became evident that the optimal concentration of Mg$^{2+}$ for the interaction of myosin A and F-actin from arterial wall was much high in compared with that from striated muscle.

Then, in order to determine which, myosin A or F-actin, was responsible for the Mg$^{2+}$ dependency for the interaction between myosin A and F-actin, Mg$^{2+}$ requirement in their interaction was investigated in cross-reaction between myosin A and F-actins from cardiac muscle and arterial wall. As shown in Fig.22, when the myosin A was the one from cardiac muscle, the superprecipitation was accelerated at low concentration of Mg$^{2+}$ (0.5 mM) regardless of any kind of F-actin, whereas it was enhanced at high concentration of Mg$^{2+}$ (10 mM) in the actomyosins syn-

Japanese Circulation Journal Vol. 37, March 1973
Fig. 19. Effect of Ca\(^{2+}\) and EGTA upon the superprecipitation of actomyosin composed of cardiac myosin A and F-actin from cardiac cold actin with or without arterial native tropomyosin. The reaction procedure was the same as that in Fig. 17 except that the concentration of MgCl\(_2\) in the reaction mixture was 5 mM instead of 10 mM. Myosin A, 0.58 mg/ml; F-actin with or without TMN, 1.40 mg/ml. In the right, the F-actin contained arterial native tropomyosin and in the left, the F-actin was without arterial native tropomyosin. The reaction in the presence of Ca\(^{2+}\) is shown by (x-x-x) and the reaction in the presence of EGTA is shown by (x-x-x-x).
Fig. 20. Effect of Mg\(^{2+}\) concentration on the superprecipitation of actomyosin synthesized from myosin A and F-actin of skeletal, cardiac and arterial muscle.

The reaction mixture: 10 mM Tris-maleate buffer (pH 7.0), 0.06 M KCl, 5 mM PEP, 1 × 10\(^{-4}\) M Ca\(^{2+}\), Py-k (10 μg), 50 μM ATP, 10 mM (the right) or 0.5 mM MgCl\(_2\) (the left). Myosin A: 0.18 mg/ml, F-actin: 0.60 mg/ml. The reaction procedure was the same as that in Fig. 17. Skeletal (-----), cardiac (X--X) and arterial actomyosin (▲—▲).

Figures are not available in this text. However, the text continues:

The similar effects of Mg\(^{2+}\)-concentration were also observed in the myosin A ATP-ase activities activated by F-actin (Fig. 21).

From the above findings, it was clarified that, the optimal concentration of Mg\(^{2+}\) in the interaction between myosin A and F-actin was much high in the case of arterial myosin A and much low in the case of myosin A from striated muscle, unrelated to origin of F-actin.

(E) Identification of filament containing actin in arterial and capillary wall

As described in (B), it has been generally accepted that F-actin forms the characteristic arrowhead structure by attachment of H-meromyosin (Fig. 14, 15) and the formation of a so-called arrowhead structure is characteristic features of F-actin filament. For identification of filaments containing actin in arterial tissue, the detection of "arrowhead structure" formed by exogenous H-meromyosin was electronmicrographically attempted in bovine mesenterial arteries by the method developed by Ishikawa. The arteries were treated with 50% glycerol as described in method in order to allow H-meromyosin to pass into arterial smooth muscle cells. The control, not reacted with H-meromyosin, was shown in Fig. 23. In this micrograph (the arterial muscular layer), we can find thick and thin filaments (designated as Thk-F and Thi-F, respectively) which are arranged parallel each other, but we can not find such Z-membranes or striated structures as observed in striated muscle. In the micrograph of the artery reacted with skeletal H-meromyosin (Fig. 24), the thin filaments demonstrated "arrowhead structure" shown as "ArHS" in Fig. 24. This finding observed in the electronmicrograph did clearly identify that the thin filaments consisted of actin. The identification seems to have not been conducted so far by others.

Furthermore, in glycerinated rat kidney, the filaments that have the similar arrowhead structure was demonstrated even in the endothelium of the capillary (Fig. 25-(b)).

**DISCUSSION**

*Japanese Circulation Journal* Vol. 37, March 1973
In reviewing the progress in the study of arterial contractile protein, it was in 1961 that Laszt et al. firstly obtained from bovine carotid artery a protein extractable in low ionic strength solution containing ATP, which showed two peaks in the ultracentrifugal pattern in the high ionic strength solution containing ATP. The two peaks corresponded to the values of sedimentation velocities of myosin A and F-actin from skeletal muscle, respectively. However, the substance they extracted, different from skeletal actomyosin, did not show any superprecipitation by ATP, and its viscosity tended to increase with elevation in KCl concentration. Then, they called this substance at “actomyosin” after the consideration that it would be related to the tonus of arterial wall. At present, it is considered that the substance they extracted contained a small amount of myosin A in comparison with the content of actin, because actin was extractable as globular actin (G-actin) in low ionic strength solution and the G-actin elevated the viscosity through the transformation to fibrous actin (F-actin) by increasing of KCl concentration.

Setting it aside, the above finding of Laszt et al. indicated that the substance resembling contractile protein of smooth muscle was, unlike the contractile protein of striated muscle, capable of dissolving in low ionic strength solution in the presence of a minute amount of ATP. This served in opening the way for research in this
Fig. 22. Effect of Mg$^{2+}$ concentration on the superprecipitation of actomyosin synthesized from myosin A and F-actin from cardiac and arterial muscle. The reaction procedure was the same as that in Fig. 20. Symbols in the figure are the same as those in Fig. 21. Myosin A: 0.18 mg/ml, F-actin: 0.60 mg/ml.

field. That is, in 1965, Rüegg$^4$ et al. obtained an actomyosin like contractile protein by eliminating the extraglobulin contaminated in the substance extracted by Lastz$^1$ et al.$^7$ from artery. This actomyosin like protein showed the superprecipitation and ATP-ase activity of the actomyosin type at low ionic strength solution containing ATP and Mg$^{2+}$$^8$ However, it did not necessarily show Ca$^{2+}$-sensitivity in the superprecipitation and the ATP-ase activity as natural actomyosin, that is myosin B, from skeletal muscle. So, we suspected that the protein extracted by Ruegg$^4$ et al.$^4$ might be a contractile protein lacking native tropomyosin like substance.

As shown in many reports$^1$,$^9$ it has been clarified that contraction of arterial specimens is inhibited in the presence of Ca$^{2+}$-chelating agents. Therefore, we considered the arterial contractile protein had to be stably respondent to Ca$^{2+}$ as well as that from striated muscle. In 1967, the extraction of the Ca$^{2+}$ sensitive contractile protein from arterial wall was attempted by us in accordance with the procedure described in this paper.$^1$,$^2$ For this, the arterial tissue was firstly treated with glycerol following the report of Ebashi$^1$ et al.$^{20}$ in which they suggested that native tropomyosin, a contractile regulatory protein respondent to Ca$^{2+}$ became more stable through the treatment with glycerol.

From 1963 to 1971, Murphy$^1$ et al.$^{1},^{22}$ also extracted contractile protein from the artery and discussed about its properties which were similar to those described in the present paper. However, the protein did not show any sensitivity to Ca$^{2+}$, and was not so sufficiently identified to be the arterial actomyosin as in our present paper.

Since 1962, Hamoir$^2$ and his coworkers$^3,^{24}$ have isolated myosin A and actin which constitute the arterial contractile protein, but many points were left unidentified with regard to the degree of purification, behaviors of the interaction of myosin and actin, and electronmicrographical structures.

In this paper, it was demonstrated in detail that the arterial contractile protein consisted of myosin A, actin and native tropomyosin respondent to Ca$^{2+}$ as the main components as previously proved in striated muscle.$^3$

As shown in results, myosin A and actin of
cardiac muscle were almost the same as those of skeletal muscle in their properties or electronmicrographic structures. However, arterial myosin A was somewhat different from myosin A of striated muscle in electronmicrographic structure of myosin aggregate and at the optimal concentration of Mg$^{2+}$ necessary for the interaction with F-actin, although any different feature was hardly found between arterial actin and actin from striated muscle in electronmicrographic structure or in the interaction with myosin A except slowness of transformation to F-actin from G-actin.

On the other hand, it is a well-known fact that cardiac muscle and skeletal muscle belong to striated muscle and arterial muscle to smooth muscle. Therefore, the above described features of arterial myosin A are thought to be noteworthy points for differentiation of the contractile protein of smooth muscle from that of striated one. In fact, we have already reported that intestinal actomyosin required at least 10 mM of Mg$^{2+}$ for the superprecipitation.$^{25}$ Filo et al. also reported that the glycerinated arterial smooth muscle filament required much more Mg$^{2+}$ for the contraction in comparison with the skeletal one.$^6$

Another interesting point demonstrated in this paper may be the fact that actin filaments were found not only in the muscular layer of artery but also in the endothelium constituting of the capillary wall. This fact may throw light on elucidation of the mechanism of microcirculation of blood.

Even in now, it is still unknown whether or not the contraction in smooth muscle might be conducted by the same mechanism as that proposed to striated muscle as “sliding theory”. However, the present paper is considered to be very contributory for physiological and pathological studies of blood circulation.

**Summary**

In order to clarify the characteristic features of arterial contractile protein, natural actomyosin (myosin B), myosin A, actin and native
tropomyosin were extracted from bovine carotid arteries and their characteristic features were compared with those from bovine cardiac muscle and from rabbit’s skeletal muscle.

In this study, the following points were clarified:

1) Arterial contractile protein mainly consisted of myosin A, actin and native tropomyosin, regulatory protein respondent to Ca\(^{2+}\) just as having been observed in those from striated muscle.

2) Arterial myosin A, actin and native tropomyosin possessed the similar features characteristic of myosin A, actin and native tropomyosin from striated muscle, respectively, as shown with following items; effect of divalent cations on ATP-ase activity of myosin A, G\(\rightleftharpoons\) F transformation in actin, the formation of “arrowhead structure” in F-actin by reaction with arterial and skeletal H-meromyosins and the demonstration of the interaction of myosin A and F-actin with regardless of their origins.

3) The optimal concentration of Mg\(^{2+}\) necessary for the interaction of myosin A and F-actin was much high (10–20 mM) in myosin A from arterial wall and very low (< 0.5 mM) in myosin A from striated muscle regardless of origin of the F-actin.

4) In electronmicrographic structures, arterial myosin aggregates were shorter in the length compared with that of striated muscle, having rod shape without tapering and possessed the fine projections only at the both ends. However, any difference has not been observed between arterial F-actin and F-actin from striated muscle which held helical structure with the pitch of about 350 Å and the width of about 80 Å.

5) The thin filaments containing actin were located not only at muscular layer of arterial wall but also at endothelium of renal capillary.

Through the above findings, it was understood that the contraction - relaxation of arterial wall, even in capillary, contributory to the performance of blood circulation was based on the association – dissociation of myosin aggregate and F-actin together with native tropomyosin respondent to Ca\(^{2+}\) through increase – decrease of Ca\(^{2+}\), which is associated with hydrolysis of

*Fig. 24. Electronmicrograph of glycerinated mesenterial artery reacted with skeletal H-meromyosin. (ArHS \(\rightarrow\)): arrowhead structure formed by reaction with H-meromyosin.*
Fig. 25. Electronmicrograph of glycerinated renal capillary.
(a) Control, without reaction with H-meromyosin
(b) Specimen reacted with skeletal H-meromyosin
The inset represents a higher magnification of the part designated by (-).
In (b), it is clear that the arrowhead structure is formed along the thin filament.

*Japanese Circulation Journal* Vol. 37, March 1973
ATP in the presence of Mg\textsuperscript{2+}.

REFERENCES


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