Mechanism of Cerebral Vasospasm **—Contractile Proteins in Smooth Muscle of** ***Bovine Cerebral Artery—***

Takuji TAKAGI, Hidekazu FUKUOKA, En-Chow TAN*,
Nobuo ITOH** and Ken HOTTA**

Department of Neurosurgery, Nagoya City Higashi General Hospital, Nagoya;
*Departments of *Neurosurgery and **Physiology,*
Nagoya City University Medical School, Nagoya

Abstract

Understanding of the pathogenesis of cerebral vasospasm requires basic study of the contraction mechanism of cerebral arteries. In this study, the morphological and biochemical properties of actomyosin from bovine cerebral arteries were examined. Arrays of thick and thin filaments were clearly seen on electron micrographs of a cross section of a muscle strip stretched to 1.5 times its normal length in Krebs-Ringer solution. The diameters of the thick and thin filaments were $110.0 \pm 19.9 \text{ \AA}$ and $51.0 \pm 8.0 \text{ \AA}$, respectively. The average ratio of thick to thin filaments was about 1:15 to 1:16. The gel pattern of Ca^{2+} -sensitive actomyosin extracted from bovine cerebral arteries revealed the presence of myosin, actin, and tropomyosin, but not skeletal troponin. The Mg-ATPase activity of actomyosin was greatly enhanced in the presence of Ca^{2+} . Rapid superprecipitation occurred after the clearing phase, when 1 mM Mg-ATP was added to a suspension of actomyosin with $10^{-5} \text{ M Ca}^{2+}$. This superprecipitation did not occur with a lower Ca^{2+} concentration of 10^{-8} M . Intracellular free Ca^{2+} appears to play a crucial role in the regulation of contraction and relaxation of cerebral arterial smooth muscle.

Key words: vasospasm, contractile proteins, bovine cerebral artery, Ca^{2+}

Introduction

Cerebral vasospasm frequently accompanies subarachnoid hemorrhage from a ruptured intracranial aneurysm. Effective treatment of cerebral vasospasm has not yet been established. This requires basic study of the contraction of cerebral arterial smooth muscle, since vasoconstriction and dilatation involve contraction and relaxation of vascular smooth muscle.

In this study, we examined the electron microscopic features of cerebral arterial myofilaments. In addition, we carried out biochemical studies utilizing contractile proteins extracted from smooth muscle of cerebral arteries.

Materials and Methods

Basilar arteries were isolated from bovine brainstems at the Nagoya City Meat Hygiene Inspection Laboratory. Specimens were prepared by helical cutting along the circular muscle ($1 \times 10 \text{ mm}$) under a dissecting microscope. Each specimen was pre-equilibrated in Krebs-Ringer solution under a resting tension of 0.5 gm for 1 hour and then fixed for 90 minutes in a mixture of 1.5% OsO_4 and 2.5% glutaraldehyde in a 0.14 M veronal buffer containing 0.046 mg/dl sucrose (pH 7.4). The tissue was dehydrated in a graded ethyl alcohol series, embedded in Epon 812, and stained with a mixture of uranyl acetate and lead citrate.²⁹⁾

Actomyosin was prepared by a modification of Ebashi's method,¹⁶⁾ as follows. The basilar artery and its tributaries were removed from 10 bovine brainstems, immersed in 50% glycerol, and allowed to stand overnight at 4°C. The glycerol was removed

Fig. 1 Procedure for the preparation of actomyosin from cerebral arterial smooth muscle.

with 10 mM tris-HCl (pH 7.0) and the arterial strips were homogenized for 30 seconds at 45,000 rpm in 5 volumes of 0.1 M KCl and 10 mM tris-HCl (pH 7.6) with a Vertis 45 homogenizer. The homogenate was centrifuged at 2000 G for 10 minutes. The precipitate and the floating layer were suspended together in 5 volumes of 0.1 M KCl and 10 mM tris-HCl (pH 7.6) containing 1% triton X-100 and again centrifuged at 2000 G for 10 minutes. The precipitate was collected and washed three times with 5 volumes of the same buffer to remove the detergent. Actomyosin was extracted from the precipitate over a 2-hour period at 4°C with 3 volumes of Guba-Straub solution (KCl 22.36 gm/l, KH₂PO₄ 13.6 gm/l, K₂HPO₄ 8.71 gm/l, pH 6.5) containing 1 mM adenosine triphosphate (ATP). The protein was purified by the conventional procedure, *i.e.*, dissolution in 0.6 M KCl followed by precipitation in 0.05 M KCl. The resulting precipitate was collected and suspended in 50 mM KCl and 20 mM tris-maleate buffer (pH 6.8). The flow chart of the procedures is presented in Fig. 1.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed as described by Weber and Osborn.³¹⁾

ATPase activity was defined as the rate of inorganic phosphate (Pi) liberation from ATP, which was measured by the method of Youngberg *et al.*³³⁾

The degree of superprecipitation of actomyosin was expressed as the increase in turbidity determined by absorbance at 660 nm using a spectrophotometer (Hitachi 139).

Protein concentrations were determined by the biuret method.¹²⁾

The free Ca²⁺ concentration in the medium was controlled with a Ca-thylene glycol-bis-(β -amino-ethyl)-tetraacetic acid buffer and calculated by the following equation:¹⁵⁾

$$pCa = 2pH - 7.28 + \log \left[\frac{(\text{EGTA}) \text{ added}}{(\text{CaCl}_2) \text{ added}} - 1 \right]$$

Fig. 2 Longitudinal section of myofilaments (MF) in bovine basilar arterial smooth muscle. Cerebral vascular smooth muscle is rich in actin filaments with dense bodies (D) corresponding to the Z-lines of skeletal muscle. Thin and thick filaments are indistinguishable. SR: sarcoplasmic reticulum, C: caveolae. Bar = 0.5 μ m.

Fig. 3 Cross section of smooth muscle stretched to 1.5 times its natural length in Krebs-Ringer solution. Thin (arrows) and thick (arrowheads) filaments can be seen. Bar = 100 nm.

Fig. 4 SDS polyacrylamide gel electrophoresis of actomyosin in cerebral arterial smooth muscle. A gel containing 10% acrylamide, 0.8% N,N-methylene-bis-acrylamide, and 0.1% SDS was subjected to electrophoresis at pH 7.2 and stained with Coomassie blue. The main components of the contractile proteins, including myosin, actin, and tropomyosin, were verified, but there was no component corresponding to skeletal troponin in this sample.

Results

I. Ultrastructure of bovine arterial smooth muscle

Myofilaments were clearly observed in an electron micrograph of a longitudinal section (Fig. 2). The sarcoplasmic reticulum, caveolae, and dense bodies were also recognized. Figure 3 shows a cross section of a smooth muscle strip stretched to 1.5 times its normal length in Krebs-Ringer solution. Arrays of thick and thin filaments measured $110 \pm 19.9 \text{ \AA}$ and $51.0 \pm 8.0 \text{ \AA}$ (mean $\pm$ SD), respectively. The average ratio of thick to thin filaments was about 1:15 to 1:16.

II. Gel patterns of actomyosin

The gel pattern of actomyosin extracted from a basilar artery and its tributaries exhibited the presence of myosin, actin, and tropomyosin, as shown in Fig. 4. Heavy and light chains of myosin A were also recognized. However, there was no component corresponding to skeletal troponin. The ratio of the actin to the myosin content was much higher than that in skeletal muscle.

III. Ca^{2+} sensitivity of actomyosin to Mg-ATPase activity and superprecipitation

Actomyosin from the basilar artery and its

tributaries exhibited Ca^{2+} sensitivity to both Mg-ATPase activity and superprecipitation, as shown in Figs. 5 and 6. The Mg-ATPase activity of actomyosin was greatly enhanced in the presence of Ca^{2+} (10^{-5} M). Thus, the specific activity of the Mg-ATPase was 2.5 nmol Pi/mg protein/min in the absence of Ca^{2+} ($< 10^{-9} \text{ M}$) and the activity was increased to 8.8 nmol Pi/mg protein/min with 10^{-5} M Ca^{2+} as shown in Fig. 5.

Superprecipitation of actomyosin from a basilar artery and its tributaries is shown in Fig. 6. When 1 mM Mg-ATP was added to a suspension of acto-

Fig. 5 ATPase activity of actomyosin from cerebral arterial smooth muscle. The reaction mixture consisted of 2 mg/ml protein, 4 mM MgCl_2 , 50 mM KCl, 20 mM tris-maleate (pH 6.8), 1 mM ATP, 25°C . The ATPase activity of the actomyosin from cerebral arterial smooth muscle was greatly enhanced in the presence of Ca^{2+} . ○: without Ca^{2+} ($\text{Ca}^{2+} < 10^{-9} \text{ M}$), ●: with Ca^{2+} ($\text{Ca}^{2+} = 10^{-5} \text{ M}$).

Fig. 6 Superprecipitation of actomyosin from cerebral arterial smooth muscle. The reaction mixture consisted of 2 mg/ml protein, 4 mM MgCl_2 , 50 mM KCl, 20 mM tris-maleate (pH 6.8), 0.5 mM ATP, 23°C . Superprecipitation of the actomyosin from cerebral arterial smooth muscle was greatly enhanced in the presence of Ca^{2+} . ○: $\text{Ca}^{2+} = 10^{-8} \text{ M}$, ●: $\text{Ca}^{2+} = 10^{-5} \text{ M}$.

myosin with 10^{-5} M Ca^{2+} , rapid superprecipitation occurred after the clearing phase. With a low concentration of Ca^{2+} (10^{-8} M), this superprecipitation did not occur.

Discussion

The study of cerebral vasospasm has a long history. In the latter half of the 1960s, experiments involving the basilar artery were numerous, and most were designed to identify substances that cause cerebral arteries to contract.^{5,23} During this period, significant knowledge was attained regarding the mechanisms of contraction and regulation of skeletal muscle at the subcellular and molecular levels, including the sliding theory¹⁴ and the Ca^{2+} -mediated regulatory system.⁷ However, studies of the mechanisms of contraction and regulation of smooth muscle, especially vascular smooth muscle, lagged behind those concerning skeletal muscle.

Unlike that of skeletal muscle, the behavior of vascular smooth muscle, particularly cerebral vascular smooth muscle, is very complicated, and its response to stimulation differs depending on the species, organ, and site. However, cerebral arteries appear to contract and relax in basically the same ways as do other muscles. The structure and function of smooth muscle reflect evolutionary processes, and smooth muscle is considered to be a link between nonmuscle cells and skeletal muscle cells in the process of differentiation. At the molecular level, contraction of vascular smooth muscle involves interaction between myosin and actin, accompanied by ATP decomposition, which resembles the process in skeletal muscle.^{24,30} Intracellular free Ca^{2+} is known to play a crucial role in the regulation of the contraction and relaxation of all smooth muscle, including cerebrovascular smooth muscle.^{10,16,28,32}

Studies of the ultrastructure of smooth muscle filaments have yielded confusing results. In 1969, Nonomura²² discovered contractile elements in smooth muscle resembling those in skeletal muscle. In 1976, Somlyo *et al.*²⁵ demonstrated the presence of thick and thin filaments in vascular smooth muscle. The difficulties encountered in demonstrating myosin (thick) filaments in smooth muscle can be ascribed to the preparatory techniques for electron microscopy (particularly because the fixation procedure to preserve the structure of the filaments inevitably causes them to swell).^{18,25,26} The block staining technique described by Nonomura is a reliable method for visualizing thick filaments.²² Although thick filaments were observed in relaxed muscle,⁶ several reports indicated that stretching of the mus-

cle before fixation facilitated the demonstration of thick filaments.^{6,11,21} Stretching the muscle to approximately 1.5 times its natural length has several advantages: it restores the muscle cells to their *in vivo* length, orients fibers along a common longitudinal axis, and facilitates the demonstration of filaments in a true cross section.²⁵ In our study, we stretched a sample specimen to approximately 1.5 times its original length in Krebs-Ringer solution and found that such stretching facilitates the demonstration of arrays of both thick and thin filaments in vascular smooth muscle. It is generally accepted that the thick and thin filaments in vascular smooth muscle correspond to myosin and actin, respectively.²¹

In 1970, Sparrow *et al.*²⁷ reported that Ca^{2+} -sensitive actomyosin from arterial smooth muscle could be extracted under conditions of low ionic strength. In 1975, Ebashi *et al.*⁹ succeeded in extracting Ca^{2+} -sensitive actomyosin from chicken gizzard smooth muscle with Guba-Straub solution; this method is much simpler and quicker than Sparrow's method. In the present study, Ca^{2+} -sensitive actomyosin from cerebral arterial smooth muscle was extracted by Ebashi's method, with a slight modification. Namely, we added the treatment with 1% triton X-100, which reduced contamination.¹⁶ With this method, approximately 5.22 mg of actomyosin was extracted from 3.48 gm of material.

The main components of actomyosin—myosin A, actin, and tropomyosin—were detected by SDS polyacrylamide gel electrophoresis, but troponin was absent. The chemical structure of the myosin of cerebral arterial smooth muscle resembled that of skeletal muscle. However, the former contained only two types of light chain, having molecular weights of 20,000 and 17,000 and corresponding to L_2 and L_3 , respectively.

Ebashi *et al.*⁹ found a component of 83,000 daltons in proteins extracted from chicken gizzard smooth muscle. Since this component, together with Ca^{2+} , activates the contractile protein, they designated it as leiotonin. In 1976, Itoh *et al.*¹⁷ discovered a component of 80,000 daltons in a regulatory protein extracted from bovine aorta by the same method as used by Ebashi and coworkers.

In mammalian smooth muscle another regulatory system, phosphorylation and dephosphorylation of the myosin light chain, participates with Ca^{2+} in the regulation of contraction and relaxation¹⁹ (Fig. 7). When the Ca^{2+} concentration is increased to more than 10^{-6} M, calmodulin-binding kinase is activated and induces an interaction between phosphorylated myosin and actin. When the Ca^{2+} concentration is decreased to lower than 10^{-7} M, liberation of

Fig. 7 Schema of the phosphorylation of the myosin light chain in mammalian smooth muscle. ATP: adenosine triphosphate, ADP: adenosine diphosphate, P_i : inorganic phosphate, P: phosphorylation.

calmodulin from kinase occurs and the kinase is inactivated. As a result, phosphatase dephosphorylates the phosphorylated light chain, thereby abolishing the interaction between myosin and actin.^{1,13} The myosin phosphorylation theory is a more plausible explanation of the Ca^{2+} regulation of smooth muscle than is the theory of an actin-associated mechanism. In any case, the regulation of smooth muscle is quite different from that of skeletal muscle. Further study of the regulation of cerebral arterial smooth muscle is necessary.

In 1969, Murphy *et al.*²⁰ reported that actomyosin ATPase activity was enhanced by a high Mg^{2+} concentration *in vitro*. The reason why a high Mg^{2+} concentration is necessary for activation of this contractile protein is still unknown, although most smooth muscle actomyosin requires a high Mg^{2+} concentration for full activation.¹⁶ Mg -ATPase activity in actomyosin extracted from a basilar artery and its tributaries increased threefold when the Ca^{2+} level was increased from 10^{-9} to 10^{-5} M.

It has been confirmed that superprecipitation of cerebral arterial actomyosin occurs when the Ca^{2+} concentration exceeds 10^{-6} M, but does not occur when it is below 10^{-8} M in the present study.

Cerebral arterial smooth muscle, as well as other types of vascular smooth muscle, contracts only in the presence of Ca^{2+} , although skeletal muscle contracts irrespective of the presence or absence of Ca^{2+} in the medium.

In 1979, Fukuoka *et al.*¹⁰ localized Ca^{2+} in bovine basilar artery by the pyroantimonate acid method and transmission electron microscopy. They docu-

mented that Ca^{2+} is bound to various intracellular structures, including the nucleus, nuclear membrane, sarcoplasmic reticulum, mitochondria, cell membrane, and caveolae of cellular invaginations. Ca^{2+} uptake by mitochondria and microsomal components extracted from smooth muscle tissue is well known.⁴ Although microsomes contain plasma membrane and internal cell membrane, their main component is sarcoplasmic reticulum. Their affinity for Ca^{2+} is stronger than that of mitochondria.^{2,3,8}

It may be reasonable to conclude that contraction and relaxation of cerebral arterial smooth muscle is regulated in a way similar to that of other vascular smooth muscle. The available data suggest that the primary event leading to cerebral vasospasm is an increase in the myoplasmic Ca^{2+} concentration in the smooth muscle cells of cerebral arteries.

References

- 1) Adelstein RS, Pato MD, Conti MA: Regulation of smooth muscle myosin kinase activity by phosphorylation and dephosphorylation, in Ebashi S, Maruyama K, Endo M (eds): *Muscle Contraction: Its Regulatory Mechanism*. Berlin, Springer, 1980, pp 303-313
- 2) Allen JC: Ca^{2+} binding properties of canine aortic microsomes: Lack of effect of cAMP. *Blood Vessels* 14: 91-104, 1976
- 3) Aoki Y, Yamashita K, Hotta K: Calcium uptake by cellular membrane from vascular smooth muscle of spontaneously hypertensive rats. *Jpn J Pharmacol* 26: 624-627, 1976
- 4) Barry KJ, Mikkelsen RB, Shucart W, Keough EM, Gavris V: The isolation and characterization of Ca^{2+} -accumulating subcellular membrane fractions from cerebral arteries. *J Neurosurg* 62: 729-736, 1985
- 5) Brawley BW, Strandness DE, Kelly WA: The biphasic response of the cerebral vasospasm in experimental subarachnoid hemorrhage. *J Neurosurg* 28: 1-8, 1968
- 6) Devine CE, Somlyo AP: Thick filaments in vascular smooth muscle. *J Cell Biol* 49: 646-649, 1971
- 7) Devine CE, Somlyo AV, Somlyo AP: Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscle. *J Cell Biol* 52: 690-718, 1972
- 8) Devine CE, Somlyo AV, Somlyo AP: Sarcoplasmic reticulum and mitochondria as cation accumulation sites in smooth muscle. *Philos Trans R Soc Lond [Biol]* 265: 17-23, 1973
- 9) Ebashi S, Toyooka T, Nonomura Y: Gizzard troponin. *J Biochem (Tokyo)* 78: 857-861, 1975
- 10) Fukuoka H, Takagi T, Nagai H, Hotta K: Calcium localization in smooth muscle of bovine cerebral artery. *J Electron Microsc (Tokyo)* 12: 5-6, 1979
- 11) Garamvelgyi N, Vizi ES, Knoll J: The regular occur-

- rence of thick filaments in stretched mammalian smooth muscle. *J Ultrastruct Res* 34: 135-143, 1971
- 12) Gornall AG, Bardwill CJ, David MM: Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 218: 653-659, 1949
 - 13) Hartshore DJ, Simankowski RF, Aksoy MO: Ca^{2+} regulation in smooth muscle and phosphorylation: Some properties of the myosin light chain kinase, in Ebashi S, Maruyama K, Endo M (eds): *Muscle Contraction: Its Regulatory Mechanism*. Berlin, Springer, 1980, pp 287-301
 - 14) Huxley HE: The mechanism of muscular contraction. *Sci Amer* 213: 18-27, 1965
 - 15) Imai S: Calcium and contraction of heart and smooth muscle. *Nature* 213: 1044-1045, 1967
 - 16) Itoh N: Ca^{2+} regulation of smooth muscle contractile proteins. *Nagoya Med J* 23: 31-59, 1978
 - 17) Itoh N, Takagi T, Hotta K: Regulatory protein of vascular smooth muscle. *J Biochem (Tokyo)* 80: 899-901, 1976
 - 18) Jones AW, Somlyo AP, Somlyo AV: Potassium accumulation in smooth muscle and associated ultrastructural changes. *J Physiol (Lond)* 232: 247-273, 1973
 - 19) Mikawa T, Nonomura Y, Ebashi S: Does phosphorylation of a myosin light chain have direct relation to regulation in smooth muscle? *J Biochem (Tokyo)* 82: 1789-1791, 1977
 - 20) Murphy RA, Bohr DF, Newman DL: Arterial actomyosin: Mg, Ca and ATP ion dependencies for ATPase activity. *Am J Physiol* 217: 666-673, 1969
 - 21) Murphy RA, Herlihy JT, Megerman J: Force-generating capacity and contractile protein content of arterial smooth muscle. *J Gen Physiol* 64: 691-705, 1969
 - 22) Nonomura Y: Myofilaments in smooth muscle of guinea pig *Taenia coli*. *J Cell Biol* 39: 741-745, 1969
 - 23) Osaka K: Experimental studies on cerebrovascular spasm in cats. *Arch Jap Chir* 38: 349-371, 1969
 - 24) Shibata N, Yamaguchi T, Yoneda H, Takeuchi K, Tanaka K, Okamura Y: Identification of myosin A, actin and native tropomyosin constituting the arterial contractile protein and their characteristics. *Jpn Circ J* 37: 229-252, 1973
 - 25) Somlyo AP, Somlyo AV: Ultrastructural aspects of activation and contraction of vascular smooth muscle. *Fed Proc* 35: 1288-1293, 1976
 - 26) Somlyo AP, Somlyo AV, Devine CE, Rice RV: Aggregation of thick filaments into ribbons in mammalian smooth muscle. *Nature New Biol* 231: 243-246, 1971
 - 27) Sparrow MP, Maxwell LC, Ruegg JC, Bohr DF: Preparation and properties of a calcium ion-sensitive actomyosin from arteries. *Am J Physiol* 219: 1366-1372, 1970
 - 28) Takagi T, Fukuoka H, Nagai H, Hotta K: Basic study on cerebral vasospasm: Ca^{2+} movement during contraction-relaxation of isolated basilar arteries. *Neurol Med Chir (Tokyo)* 19: 45-52, 1979
 - 29) Takagi T, Fukuoka H, Nagai H, Hozumi T, Hotta K, Watari N: Thin and thick filaments in the cerebral arterial smooth muscle. *J Electron Microsc (Tokyo)* 17: 457-459, 1984
 - 30) Takagi T, Nagai R, Hotta K, Itoh N: Experimental study on cerebral vasospasm: Contractile proteins in smooth muscle of bovine carotid artery. *Neurol Med Chir (Tokyo)* 18 [Part I]: 101-106, 1978
 - 31) Weber K, Osborn M: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem* 244: 4406-4412, 1969
 - 32) Yamashita K, Takagi T, Hotta K: Mobilization of cellular calcium and contraction-relaxation of vascular smooth muscle. *Jpn J Physiol* 36: 77-90, 1986
 - 33) Youngberg GE, Youngberg MV: Phosphorous metabolism; system of blood phosphorous analysis. *J Lab Clin Med* 16: 158-166, 1930

Address reprint requests to: T. Takagi, M.D., Department of Neurosurgery, Nagoya City Higashi General Hospital, 1-2-23 Wakamizu-cho, Chikusa-ku, Nagoya 464, Japan.