Basic Study on Cerebral Vasospasm : Ca²⁺ Movement during Contraction-Relaxation of Isolated Basilar Arteries

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Summary

To establish a definitive therapy for cerebral vasospasm, a basic study on the contractile mechanism of vascular smooth muscles of the cerebral artery was performed. Ultrastructural examination of smooth muscle cells in bovine basilar arteries revealed the existence of two types of myofilaments which correspond to thick and thin filaments. Also, the existence of an internal membrane system just inside of the surface membrane was confirmed.

Experiments on the mechanical activity of basilar arterial strips showed that a K⁺ induced contraction was associated with a large increase of Ca²⁺ influx from outside of the muscle cell. On the other hand, 5HT induced tension without Ca²⁺ influx. A Ca antagonist (cinnarizine or verapamil) inhibited high K⁺ induced contractions, whereas its inhibitory effect on 5HT induced contraction was small. These facts suggest that 5HT can release Ca from intracellular store sites.

The results shown here indicate that there are several kinds of bound Ca in the muscle cell, i.e.: 1) loosely; and 2) tightly bound Ca on the external surface of the cell membrane; 3) Ca bound in the internal surface of the cell membrane; and 4) Ca stored in the intracellular store sites, such as sarcoplasmic reticulum or mitochondria. It is suggested that such intracellularly immobilized Ca may play an important role in the regulation of mechanical activity of cerebral arterial muscle.

Key words: Mobilization of Ca²⁺, contraction-relaxation, myofilaments, vascular smooth muscle, cerebral artery

Introduction

Cerebral vasospasm is frequently accompanied by subarachnoid hemorrhage from a ruptured intracranial aneurysm, and effective means for its treatment have not yet been established.

Electron microscopic observations have revealed that, among the varieties of smooth muscles, there exists thick (myosin) and thin (actin) filaments and dense bodies which correspond to the Z band in skeletal muscle.^{1,5)} Myosin and actin extracted from vascular smooth muscle exhibit superprecipitation upon the addition of ATP in the presence of Mg²⁺ and Ca.^{2+ 17)} Ca²⁺ is essential for the occurrence of superprecipitation and activation of ATPase

activity of actomyosin, i.e., activity of smooth muscle is regulated by the intracellular Ca²⁺ concentration.¹⁶⁾ Actually, the regulatory protein which corresponds to the native tropomyosin of skeletal muscle, has been identified.¹⁰⁾ These facts suggest that the basic process of smooth muscle contraction involves the interaction of myosin and actin, and is regulated by the intracellular Ca²⁺ concentration, similar to that operating in skeletal muscle cells.⁷⁾

Most of the fundamental studies on smooth muscle mentioned above have been performed using chicken gizzards, intestinal, uterine and large arterial muscles. Little work has been reported on cerebral blood vessels.

In order to establish a definitive therapy for cerebral vasospasm, a basic study on

contraction-relaxation and the mechanism of regulation mediated by Ca^{2+} was performed here on the smooth muscle of a cerebral artery. Strips of basilar artery isolated from bovine were used for this purpose. We observed the ultrafine structure of muscle cells, myofibrillar filaments and internal organs, such as sarcoplasmic reticulum (SR) and mitochondria. Then, contractile activity of the artery and cellular Ca^{2+} movement (fluxes) upon various kinds of stimulation were analyzed by physiological and biochemical means. The relation between Ca^{2+} movement and contractile response was analyzed, and the mechanism of Ca^{2+} mediated regulation of muscular activity was elucidated.

Material and Methods

Preparation of sample

The basilar artery was isolated from the brain stem of a freshly slaughtered cow. For ultrastructural observation, small pieces of the artery were fixed for two hours at 0°C in a mixed solution containing 1.5% osmic acid, 2.5% glutaraldehyde and 0.046 mg per ml sucrose (pH 7.4, adjusted with veronal acetate). Dehydration of these specimens was carried out in an ethanol series and propylene oxide, and embedded in Epon 812. Sections were made by a Porter-Blume ultramicrotome NT-1, and stained in uranyl acetate for 20 minutes and then in lead citrate for the same period. These preparations were examined with a Hitachi HU-12 electron microscope. For the tension and Ca^{2+} flux measurements, a sample specimen was prepared by cutting this material helically along the circular muscle (1.12×10 mm) under a dissecting microscope. The specimen was placed in a measuring bath which contained modified Krebs solution (NaCl, 119; KCl, $4.7\text{K}_2\text{PO}_4$, 1.18; NaHCO_3 , 14.9; CaCl_2 , 1.6; MgSO_4 , 1.17; Sucrose, 50; Dextrose, 5.5 mM), equilibrated with 95% O_2 and 5% CO_2 at 35°C .

Measurement of tension

One end of the sample was fixed and the other end was connected to a force transducer (FD pick up, SB-IT, Nihon Kohden) with a thin string. After two hours pre-equilibration in Krebs solution under a resting tension of 1.5 g, isometric tensions, developed by various kinds of stimuli, were recorded by a conventional

recorder (Towa-Denpa, EPR-3T) through an amplifier (Nihon Kohden, RM-5). Muscle stimulation was achieved by replacing the medium with K^{+} rich Krebs solution (NaCl was replaced by KCl) or the addition of various amounts of noradrenaline (NA) or serotonin (5HT) solution into the medium.

Calcium fluxes

For the measurement of Ca^{2+} influx, the basilar artery was cut into small pieces (about 1 cm long) and incubated in the Krebs solution for two hours at 37°C . Then the specimen was transferred into a medium containing ^{45}Ca and drugs as stimulants. After the incubation of the sample for a certain period (usually 5, 10, 15 and 30 min), the reaction was terminated by washing the specimen for one hour with Ca^{2+} free, 10 mM HEPES buffer (pH 7.2) solution containing 10 mM LaCl_3 , which removed Ca bound to the external surface of the membrane.¹⁸⁾ Then the specimen was blotted, weighed and solubilized with a tissue solubilizer (NCS). ^{45}Ca which was taken up into the cells, was measured utilizing a liquid scintillation counter (Pakard Tricub). The efflux of Ca^{2+} was represented as the loss of ^{45}Ca which had been previously loaded into the sample in 1.6 mM Ca (^{45}Ca) Krebs solution for two hours, after the transfer of the sample into Ca^{2+} free Krebs, followed by 10 mM LaCl_3 treatment.

Results

The ultrastructure of smooth muscle cells in the bovine basilar artery

The electron microscopic appearance of smooth muscle cells in the basilar artery under normal conditions (longitudinal section) is shown in Fig. 1. There are many internal membrane-systems (sarcoplasmic reticulum) which exist inside the plasma membrane (cell membrane), particularly near the surface, distinct from the invagination of the surface membrane (caveolae). Mitochondria and Golgi complexes are observed as formed cytoplasmic elements. As the contractile elements, myofilaments and dense bodies are observed also within the cytoplasm. The filamentous structure in the muscle cell is shown in Fig. 2a (longitudinal section) and 2b (cross section). Thick and thin filaments have regular arrangements along the longitudinal axis of the

Fig. 1 Electron micrograph of smooth muscle cell of bovine basilar artery under natural condition. N: nucleus, M: mitochondria, SR: sarcoplasmic reticulum, D: dense body, C: caveolae

cell (the edge of the nucleus can be seen). The distribution of filaments is not quite as regular as in the skeletal muscle. The existence of thick and thin filaments can be clearly seen in the high magnification of the micrograph (Fig. 3). The average diameters of thick and thin filaments are 100–180 Å and 40–50 Å, respectively. The contents of the thick filaments are much less than that of the thin filaments, as observed in most of the other kinds of smooth muscles.¹¹⁾¹²⁾

Fig. 2 Longitudinal(a) and cross(b) sectional electron micrograph of smooth muscle cell. Two types of filaments can be observed.

Fig. 3 High magnification of cross section. Thick and thin filaments are randomly distributed.

Contraction induced by high K^+ depolarization, NA and 5HT in Krebs solution (Ca^{2+} ; 1.6 mM)
With the replacement of the bathing medium from the normal Krebs to an isotonic K^+ (125 mM) Krebs solution, the basilar artery developed large tension, as shown in Fig. 4a. NA, on the other hand, caused only small responses of the muscle even at high dosages, such as $10^{-5}M$ (Fig. 4b). The strip responded very sensitively upon stimulation of 5HT, as shown in Fig. 4c. Low sensitivity to NA and high sensitivity to 5HT seems to be one of the characteristics of the basilar artery.

Contraction in a Ca^{2+} free medium

Tension that had developed by high K^+ in normal Krebs was completely abolished in the absence of Ca^{2+} (0.1 mM EGTA), as shown in Fig. 5b. The presence of verapamil (a Ca^{2+} antagonist) also inhibited K^+ induced contraction nearly to zero even in the presence of Ca^{2+} in the medium (Fig. 5c). These results indicate that K^+ induced contraction requires the presence of Ca^{2+} in the medium. On the other hand, the reduction of 5HT induced tension was very small in the Ca^{2+} free medium after one min. incubation (Fig. 5e). Basilar artery still developed approximately 1/3 of original tension after 15 min. incubation in the Ca^{2+} free medium (Fig. 5f) upon 5 HT stimulation. Inhibitory action of verapamil ($10^{-6}M$) on 5 HT

Fig. 4 Recording of mechanical responses induced by a) high K^+ , b) NE and c) 5HT in normal Krebs solution. Stimulants and their concentrations are indicated in the figure.

induced tension was much weaker than in the case of the K^+ induced contraction (Fig. 5g).

The effect of cinnarizine on the relaxation of the basilar artery

The effect of cinnarizine on the contraction of the basilar artery induced by high K^+ and 5HT was examined in various concentrations of Ca^{2+} . The specimen was incubated in a Ca^{2+} free medium containing $10^{-6}M$ cinnarizine for 20 min and then a stimulant (high K^+ or 5HT) was added. Ca^{2+} was added stepwise to this medium and the maximum tension development at each Ca^{2+} concentration was recorded. As shown in Fig. 5, 5HT induced contraction of the basilar artery was enhanced with an increase of external Ca^{2+} concentration (0 mM–5 mM). The presence of $10^{-6}M$ cinnarizine abolished the K^+ induced contraction almost completely except in the very high Ca^{2+} concentration range (above 3mM). 5HT induced contractions were also inhibited by cinnarizine, although to a much lesser extent. 5HT can induce tension even at zero Ca^{2+} and with the presence of cinnarizine in the medium, as shown in Fig. 6. Similar results were obtained with varapamil (data is not shown).

Ca^{2+} fluxes

Ca^{2+} influxes, represented as ^{45}Ca retained by the specimen after La^{3+} treatment, occurred biphasically in the control medium, viz., a rapid initial phase and a subsequent slow one. High K^+ in the medium accelerated the rate of Ca^{2+} influx in both phases markedly, as shown in Fig. 6a. When the artery was preincubated in the medium containing cinnarizine, the rate of K^+ induced enhancement of Ca^{2+} influx was reduced to the control level (Fig. 7a). 5HT had no appreciable effect on the Ca^{2+} influx and efflux across the cell membrane of the basilar artery. The Ca^{2+} efflux, measured as the loss preloaded ^{45}Ca , was reduced by the high K^+ (Fig. 7b), viz., K^+ accelerates and decelerates the rate of Ca^{2+} influx and efflux, respectively, of the arterial cell, while 5HT gave no effect on Ca^{2+} fluxes.

Discussion

Myosin has been isolated from vascular smooth muscle by chemical means, and the individual molecules of myosin so obtained have essentially the same structure and functions as those from striated muscle.¹⁷⁾ Although several reports

Fig. 5 Mechanical response of basilar artery induced by high K^+ (125 mM) and 5HT ($5 \times 10^{-6} M$) in Ca^{2+} free Krebs solution. a) control, tension developed by K^+ in normal Krebs solution. b) tension in Ca^{2+} free medium (0.1 mM EGTA). c) tension development, effect of verapamil in normal Krebs. d) tension developed by 5HT in normal Krebs solution. e) tension in Ca^{2+} free medium after one min incubation. f) tension in Ca^{2+} free medium after 15 min incubation. g) tension development, effect of verapamil in normal Krebs.

Fig.6 Maximum tension induced by high K^+ and 5HT in Krebs solution at various concentration of Ca^{2+} ; effect of cinnarizine ($10^{-6} M$)
 ○ 5HT $5 \times 10^{-6} M$, ● 5HT + cinnarizine ($10^{-6} M$)
 △ KCl 125 mM, ▲ KCl + cinnarizine ($10^{-6} M$)

which suggest the presence of two types of filaments (thick and thin) have appeared, it is difficult to demonstrate clearly their presence in vertebrate smooth muscle morphologically, probably due to the difficulty of fixing or staining. Nonomura¹¹⁾ clearly showed the presence of two types of myofilaments in smooth muscle of guinea pigs' taenia coli with block

Fig. 7 Calcium fluxes of smooth muscle of basilar artery, effect of high K⁺, 5HT and cinnarizine. a) influx, b) efflux

○ control, □ 125 mM K⁺, △ 5 × 10⁻⁶ M 5HT,
▲ 125 mM K⁺ + 10⁻⁶ M cinnarizine

staining, which might be the most suitable method for this purpose. The sizes of thin and thick filaments reported by Nonomura were 40–70 Å and 120–170 Å, respectively. Schoenberg¹⁴⁾ reported the elimination of thick filaments by prolonged washing and glycerination. Panner and Honing¹³⁾, who have shown the presence of two types of myofilaments in turkey gizzard, have suggested that; 1) myosin exists as small functional units; and 2) the contraction mechanism of smooth muscle is that actin filaments interdigitate and slide with respect to one another, driven by small units of myosin. Our observations have revealed the existence of two types of filaments in the smooth muscle of the basilar artery under normal conditions. These facts suggest the possibility of sliding between thick and thin filaments along the longitudinal axis of vascular smooth muscle, as well as in the other types of smooth muscle.

Regulation of the activity of contractile protein of smooth muscle is achieved by Ca²⁺ and regulatory protein i.e., contraction of smooth muscle is controlled by myoplasmic

Ca²⁺ concentration. The mechanical response of the basilar artery to high K⁺ depolarization requires the presence of Ca²⁺ in the medium. Acceleration of Ca²⁺ influx by K⁺ shown in this experiment indicates that upon depolarization, Ca²⁺ in the medium as well as that bound to the outer surface of the cell membrane enter into the cell. Both K⁺ induced contraction and Ca²⁺ influx were inhibited by cinnarizine (a Ca²⁺ antagonist). Therefore, it may be concluded that Ca²⁺ entering from the outside of the cell is responsible for the K⁺ induced contraction. On the other hand, 5HT induced tension of the basilar artery is reduced only to a small extent in a Ca²⁺ free medium or in the presence of cinnarizine or verapamil. Also, 5HT gave no effect on Ca²⁺ fluxes across the cell membrane (Fig. 7). These facts suggest that the activator Ca²⁺ for 5HT induced contraction is released from the intracellular store sites.

Several workers have shown morphologically and biochemically the existence of Ca²⁺ binding sites in smooth muscle cells, such as in the internal surface of the cell membrane^{1,3,20)} and

in the SR and mitochondria.^{1,3,4,5,6,16)} It is shown here that there exists an internal membrane system which may act as Ca store sites in cerebral arterial muscle cells (Fig. 1). Isolated cell membranes and SR from a variety of smooth muscle exhibit Ca accumulating ability in the presence of ATP and Mg.^{2+ 9,19)} Thus, 5HT induced contraction of the basilar artery might reflect the Ca retained in the intracellular sites.

The results obtained in the present experiments and in our previous report²⁰⁾ suggest that there exists several kinds of bound forms of Ca in the cell membrane of smooth muscle and immobilized Ca in the intracellular sites, viz.: 1) loosely bound Ca on the external surface of the membrane (The amounts of this Ca depends directly on the Ca^{2+} in the medium and can be removed by lowering the external Ca^{2+} concentration); 2) tightly bound Ca which cannot be easily removed by low Ca^{2+} in the medium, but is removed by La^{3+} treatment; 3) Ca which is not removed by La^{3+} but can be mobilized by the action of certain agents (NE, caffeine and other vasoactive drugs), (Possibly this Ca^{2+} is localized on the internal surface of the cell membrane); and 4) Ca stored in intracellular sites, such as SR, (This Ca is released by 5HT or caffeine; exchange between this Ca and extracellular Ca^{2+} is a slow process). Utilization of activator Ca from these cellular Ca pools differs depending on the kinds of stimulation as well as the kinds of muscle cells.⁶⁾ The low sensitivity of the basilar artery to NE, which induces a large response in aortic muscle^{4,20)} may be attributed to the lack of Ca pools in the cell membrane of the basilar artery. Therefore, it seems that intracellularly stored Ca plays an important role for controlling the tone of the basilar artery.

Ca antagonists (cinnarizine or verapamil) block Ca entry into the cell from outside, but they may have no direct effect on the intracellularly stored Ca. However, the level of intracellularly stored Ca is controlled by the external Ca concentration at a steady state, although the exchange between intracellularly stored and extracellular free Ca^{2+} may be slow. Therefore, the presence of a Ca antagonist in the medium results in the reduction of stored Ca. Administration of a Ca antagonist may be quite useful for the prevention of cerebral vasospasm

because it inhibits the contraction of smooth muscle directly by blocking Ca^{2+} entry from outside of the cell and also reduces muscle tone by reducing the amount of intracellularly stored Ca.

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