Science Journal of Kanagawa University **31** : 1-10 (2020)

■ Full-Length Paper ■

Physiological and Ultrastructural Studies on the Origin of Activator Calcium in Body Wall Muscles of Spoon Worms

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Abstract: To examine the origin of activator Ca and its translocation during contraction in body wall muscles (BWM) of spoon worms, *Urechis unicinctus*, physiological and ultrastructural studies, including cytochemistry, were performed. The potassium (K-) contracture tension was significantly reduced by the removal of external Ca, and by the application of Mn, La and verapamil. On the other hand, caffeine induced a prolonged contraction. The removal of Ca and Mg from the external solution, and the rapid cooling caused an irregular or oscillatory contraction. These results suggested that, in BWM fibers, the activator Ca is supplied partially from both external solution and intracellular Ca-accumulating structures. Ultrastructural observations revealed that the muscle fibers contain a relatively large amount of sarcoplasmic reticulum (SR). The fractional volume of the SR relative to the fiber volume was 2~5% in all fibers of three muscle layers. To demonstrate the Ca localization, the muscle fibers were fixed by pyroantimonate (PA) methods at resting and contracting states. In the resting fibers, the PA precipitates were exclusively localized in the SR and the inner surface of plasma membrane. On the other hand, in the contracting fibers, they were diffusely distributed in the central regions of myoplasm, and had disappeared from the SR and plasma membrane. X-ray microanalysis revealed that the PA precipitates contain Ca. With the results of physiological experiments, these results indicate that the activator Ca originates not only from the external solution, but also from the intracellular Ca-accumulating structures, the SR and the inner surface of plasma membrane.
Keywords: regulatory mechanism of smooth muscle, intracellular Ca localization, Ca translocation during contraction, body wall muscles of spoon worms, pyroantimonate method

Introduction

It has been generally accepted that the mechanical activity in muscles is regulated by the change of free Ca concentration in the myoplasm[1)]. However, the sources of Ca ions that activate the contractile mechanism in smooth muscles remain to be determined because both vertebrate and invertebrate smooth muscles exhibit considerable functional and structural variations. The peristaltic movement traveling along the entire body of spoon worms is effectively performed by contraction of body wall muscles (BWM). Although pharmacological investigations have provided information about the

neurotransmitters regulating muscle contraction[2], little information is available about the fine structures and their role in excitation- contraction coupling in the BWM of spoon worms. Furthermore, it has been reported that the body wall (BW) is constructed by three muscle layers; the inner circular, middle longitudinal, and outer circular layers[3]. However, the physiological properties and structural features have not been compared among these muscles.

Numerous cytochemical methods have been established to detect the intracellular Ca localization in various types of muscles. The pyroantimonate (PA) method is now considered the best because it can demonstrate both intracellular Ca localization and Ca translocation during muscle contraction[4-10]. Using the PA method with physiological experiments, the present study was performed to clarify the intracellular Ca localization and translocation during mechanical activity in the BWM fibers of spoon worms.

When the body of spoon worms shrinks in response to injurious stimulation, the contraction of BWM is sometimes prolonged. This mechanical response of BWM closely resembles the catch found in molluscan smooth muscles, e.g. the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*[11]. In ABRM fibers, the thick filament protein twitchin was reported to regulate the catch[12-14]. A preliminary immuno-electron microscopic study was also performed to examine the existence of twitchin in the BWM fibers of spoon worms.

Materials and Methods

Preparation

Spoon worms (relaxed body length, ~20 cm), *Urechis unicinctus*, were obtained from a commercial source and kept in circulating seawater at 18℃ (Fig. 1A). The preparations of muscle fiber bundles were obtained from three layers of the BW (Fig. 1B), containing inner circular body wall muscle (ICBWM), longitudinal body wall muscle (LBWM) and outer circular body wall muscle (OCBWM). To prepare the ICBWM and OCBWM, animal bodies were cut transversely to give ring strips of approximately 2 mm in width. These ring strips were cut along the middle longitudinal layer to separate the inner and outer circular layers, and then muscle fiber bundles of 10-20 mm in length were prepared. In the preparation of LBWM, the animal body was dissected longitudinally along the median line, and muscle fiber bundles of 2 mm in width and 10-20 mm in length were isolated.

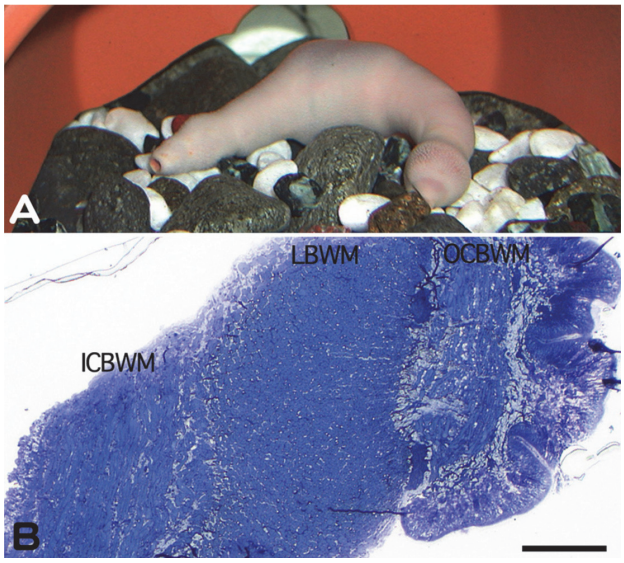

Fig. 1. Spoon worm and its body wall construction. A. Spoon worm living in a water tank filled with circulating ASW. B. Light micrograph of the section cut transversely to the body, showing three types of body wall muscles (BWM); inner circular BWM (ICBWM), longitudinal BWM (LBWM) and outer circular BWM (OCBWM). Scale bar, 0.1 mm.

Physiological experiments

A pair of stainless-steel wire connectors was tied to both ends of the preparation with a cotton thread. Then, the preparation was mounted horizontally at its slack length in a chamber filled with experimental solution; one end of the preparation was attached to the mechanical support and the other end was connected to a strain gauge (U-gauge, Shinko Tushin Co.) to record the isometric tension on an ink-writing oscillograph. The standard experimental solution was artificial seawater (ASW) having the following composition (mM): NaCl, 513; KCl, 10; $CaCL_2$, 10; $MgCl_2$, 50 (pH adjusted to 7.2 by $NaHCO_3$). The preparation was mainly stimulated to contract by high K solution, prepared by substituting K ions (20-400 mM) for an equal amount of Na ions in ASW. Ca-free and Ca- and Mg-free (Ca・Mg-free) solutions were prepared by removing these ions and adding an osmotically equivalent amount of Na ions in ASW.

Furthermore, to remove Ca ions completely, 2 mM ethyleneglycol-bis(2-aminoethylether)-N,N, N', N' -tetraacetic acid (EGTA) was added to ASW. Other

experimental solutions used to assess the effects of drugs and ions on the mechanical response were made by addition of the drugs at appropriate concentrations to the standard solution and/or by substitution for Na. The physiological experiments were performed at room temperature.

Electron microscopy

For conventional electron microscopy, relaxed preparations were prefixed with a 2-6% glutaraldehyde solution containing 2 mM $CaCl_2$ (pH 7.2 by 0.1 M cacodylate buffer). Then, they were postfixed in a 2% OsO4 solution (unbuffered), dehydrated with a graded series of ethanol, substituted by propylene oxide, and embedded in Quetol 812. Ultrathin sections of fixed specimens were cut on an Ultracut N ultramicrotome (Reichert, Vienna, Austria), and stained with uranyl acetate and/or lead citrate. Both stained and unstained sections were examined by using a transmission electron microscope (JEM2000EX: JEOL, Akishima, Tokyo, Japan).

To estimate the volume of sarcoplasmic reticulum (SR), digital electron micrograph images of BWM fibers were analyzed by the montage method[15-19] using image analysis software (Image J). Cross-sectional areas of a specified fiber on an electron micrograph and total cross-sectional area of all SR included in the specified fiber were measured in μm^2, and the fractional volume of SR, i.e. the total SR volume relative to the fiber volume was calculated based on the assumption that the value of the area is equivalent to the value of volume.

For cytochemistry to demonstrate the intracellular Ca localization and translocation during muscle contraction, the PA method[5-10] was applied. Preparations of BWM fiber bundles were fixed with 2% OsO_4 solution containing 2% potassium pyroantimonate (K-PA: $K[Sb(OH)_6]$), dehydrated with ethanol, and embedded in Quetol 812. Calcium in PA precipitates was identified in the sections without staining by using an energy dispersive X-ray microanalyzer (JEM1230 TEM with EX-14033JTP detector; JEOL, Akishima, Tokyo, Japan).

For immuno-electron microscopy to examine the existence of twitchin, relaxed preparations were fixed with 4% paraformaldehyde (pH 7.2 by 0.1 M phosphate buffer) and treated with 50 mM glycine solution (pH 7.2 by 0.1 M phosphate buffer) to neutralize the aldehyde groups. After dehydration with an ethanol series on ice or at -20℃ , they were embedded in Lowicryl K4M resin, and polymerized by illumination with ultraviolet light at -20 ℃ . Ultrathin sections treated previously with 20 mM glycine and 1% bovine serum albumin (BSA) were immunostained with anti-twitchin antibody (anti D2 antibody)[13,14], and further stained with 10 nm colloidal gold conjugated antibody. Immuno-stained sections stained further with or without uranyl acetate and lead citrate were examined by using an electron microscope (JEM2000EX; JEOL, Akishima, Tokyo, Japan).

Results

Mechanical properties

The muscle fiber bundles of the BW were caused contraction by increasing the external potassium concentration ([K]o). The mechanical response was tonic in character, as shown in typical examples of isometric tension records in Fig. 2. The mechanical threshold for isometric tension rise was approximately 20 mM K. The isometric contracture tension was related to the log of [K]o by an S shaped curve in the three types of BWM (Fig. 3). The peak tension reached its half-maximum at approximately 40-50 mM K, and full maximum at 200 mM K. Thus, the contracture induced by 200 mM of [K]o (abbreviation, 200K) was used as a control to examine the effects of drugs and ions on the potassium (K-) contracture. The response to [K]o was slightly different among the three types of BWM. The fiber bundle of

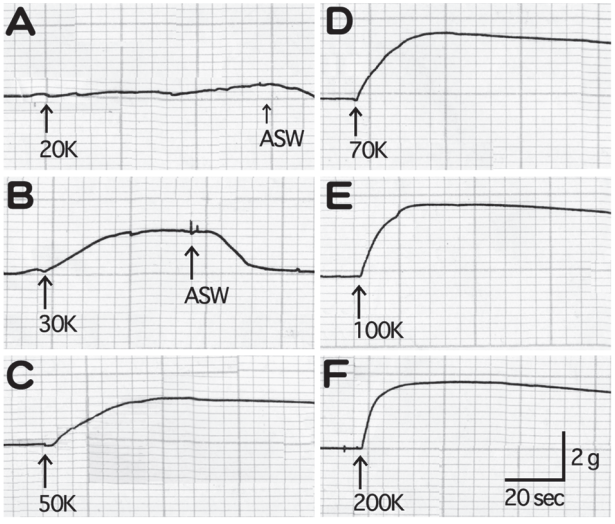

Fig. 2. Examples of the mechanical response to different values of high [K]o in ICBWM. A-F. Labels (20K, 30K, 50K, 70K, 100K, and 200K) indicate [K]o in mM.

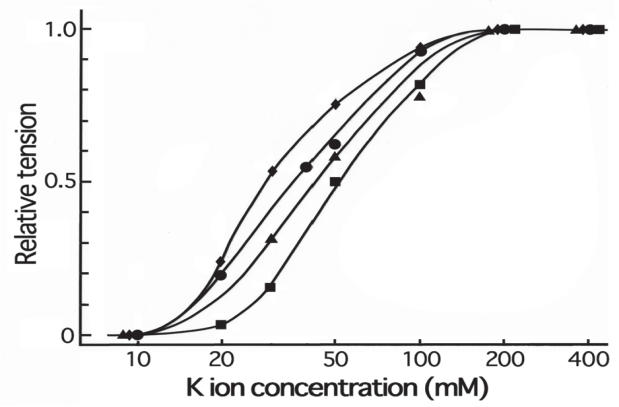

Fig. 3. Relationships between the peak tension of K-contracture and the external potassium concentration, showing S-shaped curves. Solid circles and diamonds for ICBWM, solid squares for LBWM, solid triangles for OCBWM. Solid diamonds indicate the curve in 20 mM Ca. Tension is expressed relative to the maximal tension. Abscissa indicates the logarithmic potassium concentration.

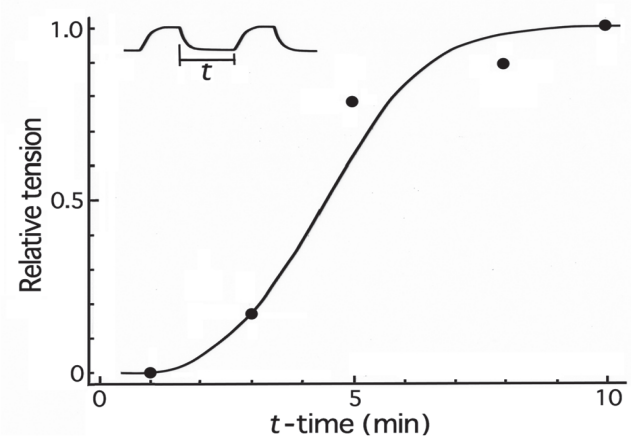

Fig. 4. Recovery of contractile ability in the K-contracture of ICBWM. The extent of recovery is expressed as the magnitude of the second (test) contracture, and t-time indicates the resting time in ASW between first (control) and second (test) contractures (see inset).

ICBWM was the most sensitive, whereas the fiber bundle of LBWM was the most insensitive to [K]o. When the external concentration of Ca ([Ca]o) was increased from 10 to 20 mM, the S-shaped curve for ICBWM was slightly shifted to a lower potassium concentration, with a decrease in the mechanical threshold.

The mechanical response induced by ACh (10^{-7} to 10^{-1} M) was also examined in ICBWN and LBWM, and a similar S-shaped curve demonstrated the relationship between isometric tension and ACh concentration, with a tension rise at 10^{-6} to 10^{-5} M and maximum tension at 10^{-3} to 10^{-1} M.

Prior to the experiments on factors influencing K-contracture, the recovery time of contractile ability in ASW following the first contracture (control contracture) with 200K was measured. As shown in Fig. 4, 10 min was sufficient for recovery. Thus, in this study, a resting time of 10 min was aaplied before the test contracture.

Factors influencing K-contracture

When Ca ions were removed from the external solution by preloading of Ca-free ASW for 10~20 min, the contracture tension in response to 200K was reduced by ~25% in ICBWM, and by 70~80% in LBWM and OCBWM (Fig. 5). As shown in Fig. 3, the K-contracture in ICBWM was slightly enhanced by increasing [Ca]o from 10 to 20 mM, with a decrease in the mechanical threshold.

In ICBWM, Mn and La ions reduced the tension to

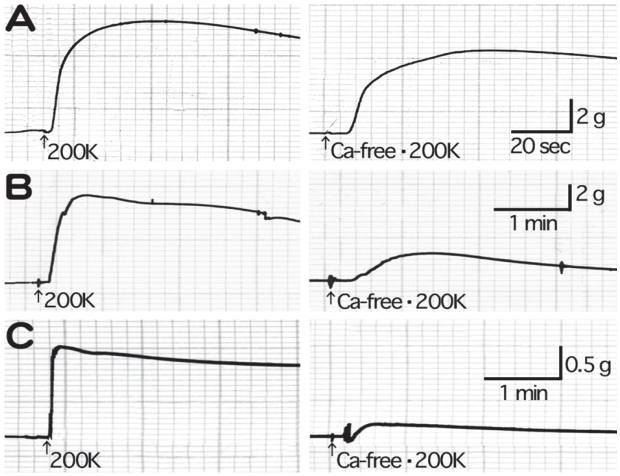

Fig. 5. Effects of Ca removal from external solution on the K-contracture. Contracture tension in ICBWM (A), in LBWM (B), and in OCBWM (C). Left records indicate control contractures and right records indicate test contractures.

approximately 75% of the maximum tension induced by the control contracture (Fig. 6A, B). Verapamil, a Ca-channel blocker, reduced the tension to 35~40% of the maximum contracture tension (Fig. 6C).

Mechanical responses induced by the release of intracellular Ca

After preloading of Ca-free solution containing 2 mM EGTA for 10-20 min, BWM fiber bundles developed a prolonged contracture tension when 1 mM caffeine was applied. The caffeine contracture tension was approximately 43% of the maximum K-contracture tension in ICBWM (Fig. 7A), although it was small, being approximately 1% of the maximum

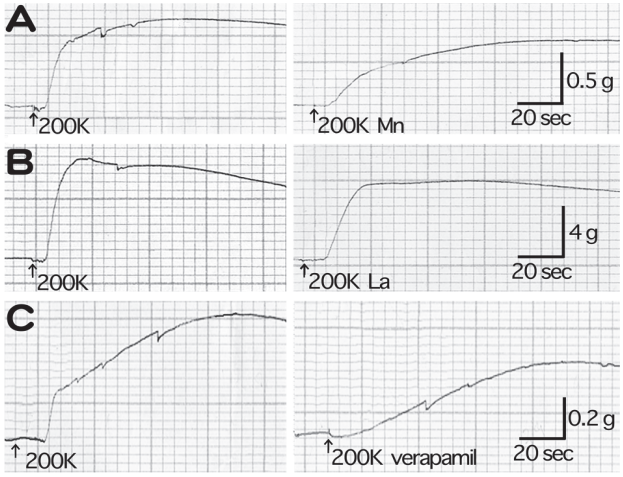

Fig. 6. Effects of Ca-influx interruption on the K-contracture in ICBWM. Development of contracture tension is repressed by the application of 10 mM Mn (A), 1 mM La (B), and 1 mM verapamil (C). Left records indicate control contractures and right records indicate test contractures.

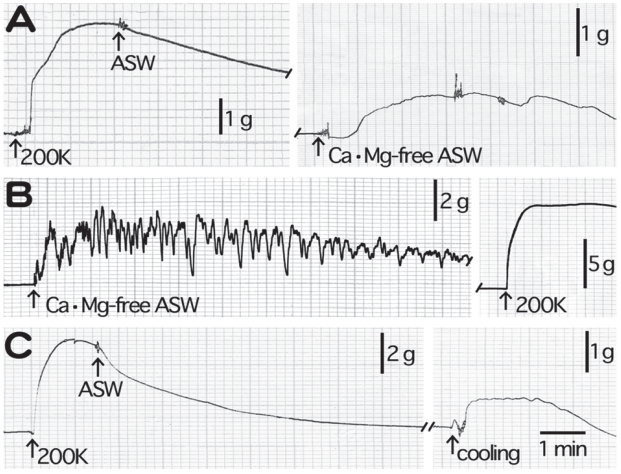

Fig. 8. Mechanical responses of BWM fiber bundles to factors causing intracellular Ca release. Mechanical responses to the application of Ca- and Mg-free solution (Ca・Mg-free ASW) in ICBWM (A) and LBWM (B), and to rapid cooling of ICBWM (C).

K-contracture tension in LBWM (Fig. 7B).

After recovery from the control K-contracture by soaking in ASW, Ca・Mg-free solution was applied (Fig. 8A, B). The BWM fiber bundles exhibited irregular or oscillatory development of tension, amounting to approximately 18% and 28% of the maximum K-contracture tension in ICBWM (Fig. 8A) and LBWM (Fig. 8B), respectively. On the other hand, when the fully restored ICBWM fiber bundles were rapidly cooled by changing the temperature of ASW from ~20 ℃ to 5-3 ℃ within 5-10 sec, a small contracture was induced (Fig. 8C). The rapid cooling contracture tension was approximately 20% of the maximum tension of control K-contracture observed at room temperature.

Fine structures of BWM fibers

Prior to electron microscopy, transverse thick sections of the body wall, stained with toluidine blue, were observed by a light microscope. As shown in Fig. 1B, the BW was constructed by three muscle layers, with a thickness of approximately 0.2, 0.35, and 0.08 mm for ICBWM, LBWM, and OCBWM, respectively. In these muscle layers, homogeneous muscle fibers were packed compactly.

Transverse ultrathin sections revealed that the BWM fibers were uninucleated and unstriated muscle fibers of 5-15 μm in diameter, and contained thick (diameter, ~30 nm) and thin (diameter, ~7 nm) myofilaments (Fig. 9). The central region of myoplasm was occupied by these contractile elements. The sarcoplasmic reticulum (SR) consisted of vesicular elements in variable size and shape, and was localized exclusively near or beneath the plasma membrane. They frequently exhibited characteristic features of diads by forming junctions with the plasma membrane. As shown in Table 1, average values of fractional SR volumes relative to the fiber volume were 3.9% in ICBWM, 5.2% in LBWM, and 2.1% in OCBWM. Mitochondria were frequently found in the peripheral region of fibers and/or near the SR. Caveolae, plasma membrane invaginations, were not found in these muscle fibers. These structural features were common among muscle fibers of all three BWM layers.

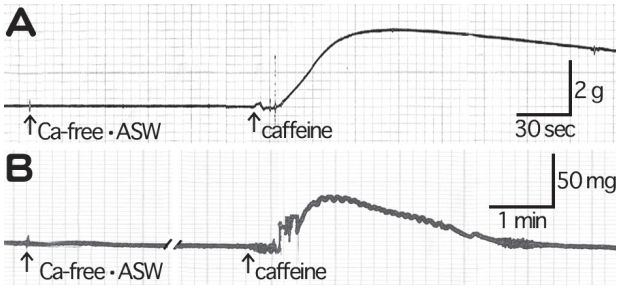

Fig. 7. Mechanical responses of BWM fiber bundles to caffeine (1 mM). After preloading of Ca-free ASW, contractures were induced by caffeine in ICBWM (A) and LBWM (B).

Table 1. Fractional volumes of the SR relative to the fiber volume in body wall muscles of spoon worms

ICBWM	LBWM	OCBWM
3.9 ± 4.1	5.2 ± 3.0	2.1 ± 1.7
(n=14)	(n=11)	(n=12)

Values (%) are the mean±SD.

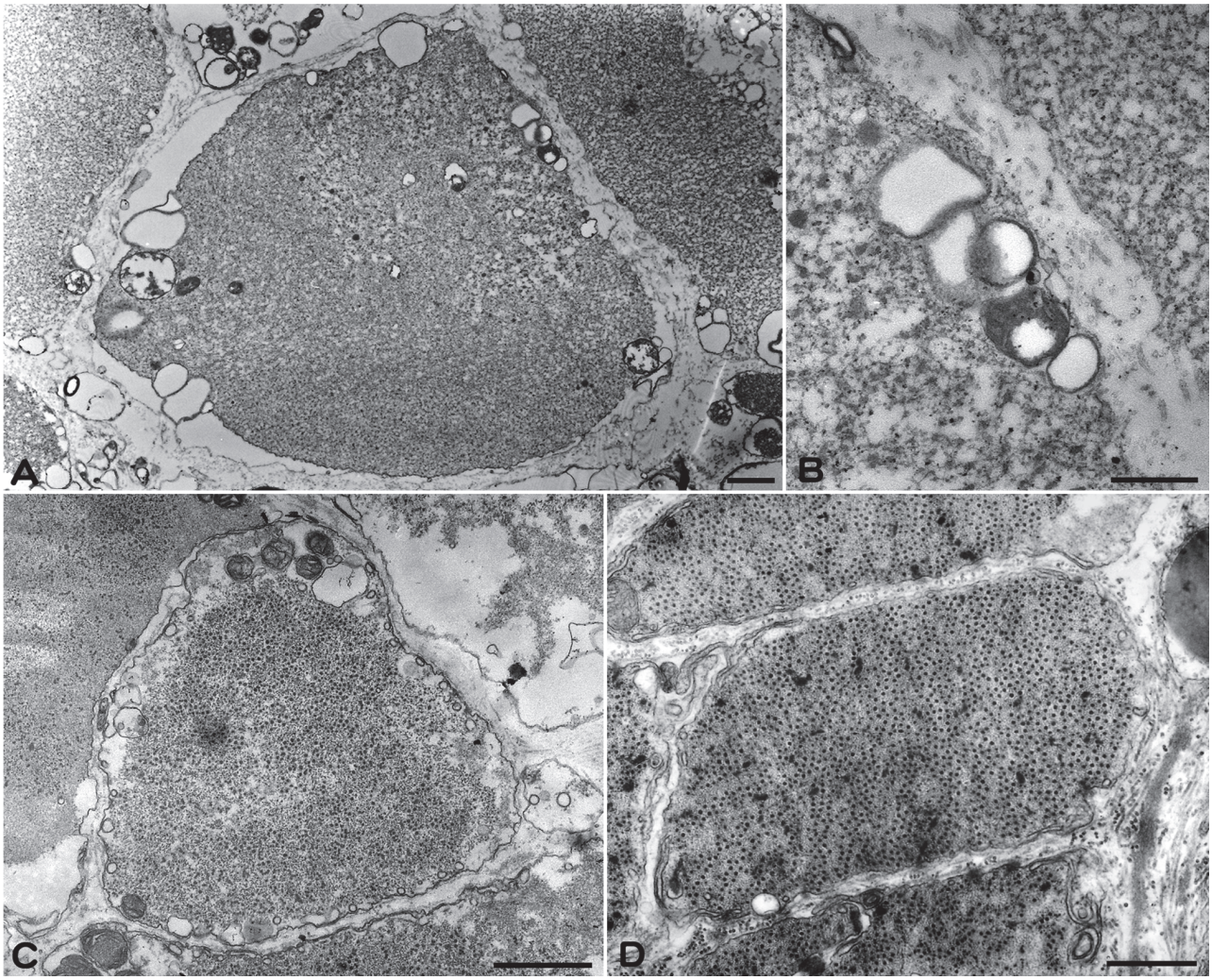

Fig. 9. Fine structures of BWM fibers. A. Transverse section image of ICBWM fibers, showing the localization of SR beneath the plasma membrane. B. Enlarged view of SR localization in Fig. 9A. C. Transverse section of LBWM fibers. D. Transverse section of OCBWM fibers. Scale bars, 1 μm (A, C, D) and 0.5 μm (B).

Intracellular Ca localization and translocation during contraction

Fiber bundles of BWM were chemically fixed with 2% OsO_4 solution containing 2% potassium pyroantimonate (PAOs) to demonstrate the intracellular Ca localization and translocation during muscle contraction. Isometric tension records during the course of cytochemical fixation are shown in Fig. 10. When the relaxed fiber bundles were fixed after the complete recovery in ASW, no significant increase in tension was observed in ICBWM (Fig. 10A), demonstrating successful fixation at the resting state. Similar tension records were obtained in LBWM and OCBWM. On the other hand, in fiber bundles of BWM contracted with 200K, the PAOs solution was applied near the peak of contracture tension (Fig. 10B-D). After the application of PAOs, the tension in the three types of BWM gradually decreased. How-

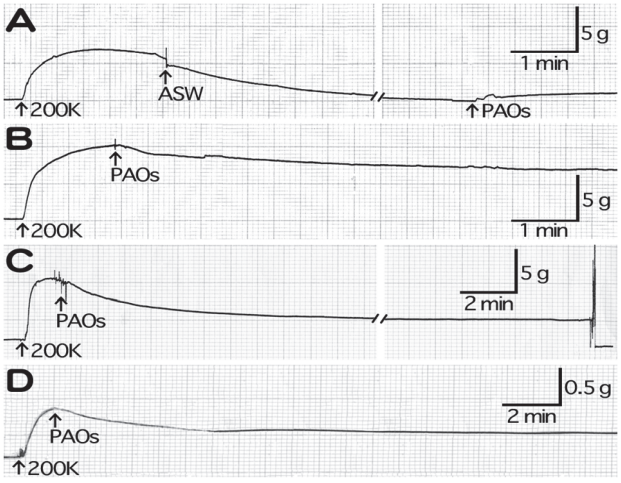

Fig. 10. Tension changes in BWM fibers during fixation with pyroantimonate-OsO4 (PAOs) solution. For the resting state, fibers were fixed after relaxation of a control K-contracture (A). For the contracting state, fibers were fixed at the tension peak of the control K-contracture (B, C, D). Typical records of ICBWM (A, B), LBWM (C), and OCBWM (D).

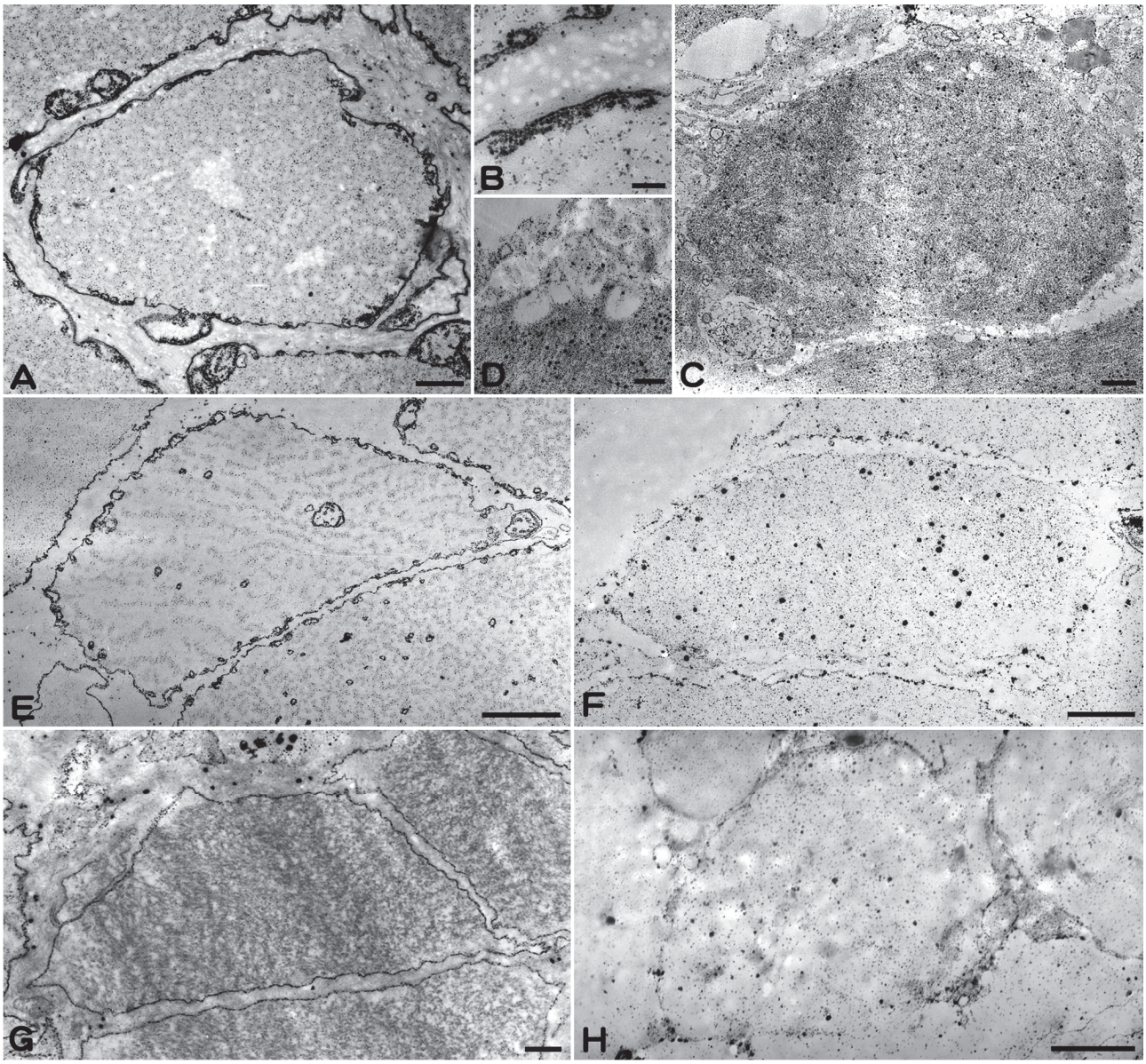

Fig. 11. Intracellular localization of electron-opaque PA precipitates in BWM fibers. A-D. Cross-sections of ICBWM fibers. E, F. Cross-sections of LBWM fibers. G, H. Cross-sections of OCBWM fibers. Precipitates are found exclusively at the peripheral region of resting fibers (A, E, G), whereas they are diffusely distributed in the myoplasm of contracting fibers (C, F, H). High-magnification view of ICBWM fibers reveals the localization of precipitates in the SR and along the inner surface of plasma membrane in the resting state (B), and the conspicuous decrease in precipitates in the peripheral region in the contracted state (D). Scale bars; 1 μm (A, C, E-H), 0.2 μm (B) and 0.5 μm (D).

ever, the tension was well maintained at a relatively higher level, demonstrating successful fixation in the contracted state.

In the resting state of all BWM fibers, electron-opaque PA precipitates were mainly found at the peripheral region of fibers (Fig. 11 A, E, G). Closer examination of the fiber periphery revealed that PA precipitates were located along the inner surface of the plasma membrane and SR limiting membrane (Fig. 11B). However, few or no precipitates were found in the central region of the myoplasm and extracellular space. On the other hand, in all BWM fibers fixed during K-contracture, PA precipitates were diffusely distributed in the central region of the myoplasm where myofilaments were packed closely (Fig. 11C, F, H). In contrast, PA precipitates conspicuously decreased at the inner surface of the plasma membrane and in the SR lumen, suggesting the intracellular movement of Ca to the central region of the myoplasm (Fig. 11D).

The electron-probe X-ray microanalysis was performed to confirm the existence of Ca in the PA precipitates. The X-ray spectra from the precipitates localized at the plasma membrane and the SR in the

resting fibers, and at the myoplasm in the contracting fibers always exhibited the most distinct peak at 3,620 eV (Fig. 12), considered to be a combination peak of antimonite and calcium (Sb-Ca)[4-7]. Relative concentration ratios of elements were calculated for the X-ray spectra obtained from the PA precipitates found in ICBWM fibers (Table 2), and indicated the existence of Ca in the PA precipitates.

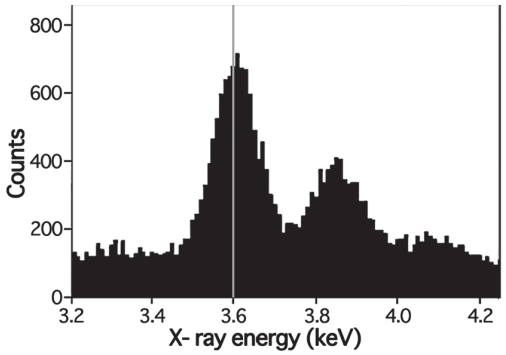

Fig. 12. A typical example of X-ray spectra obtained from the PA precipitates found in the SR of resting ICBWM fibers. The vertical gray line indicates the position of Sb-Lα emission at 3,600 eV. Note the distinct peak at 3,620 eV (Sb-Ca combination peak). Peaks of Sb-L$_{\beta 1}$ (3,840 eV) and SB-L$_{\beta 2}$ (4,100 eV) are also shown. The ordinate gives the number of X-ray events, and the abscissa shows the X-ray energy in keV (range 3.2 ~ 4.2 keV).

Immuno-electron microscopy on the existence of twitchin

As a preliminary examination to demonstrate the existence of twitchin in BWM of spoon worms, immuno-electron microscopy was performed. When transverse sections from fiber bundles of ICBWM were immuno-stained with anti-twitchin antibody, gold particles as markers of twitchin localization were noted on thick filaments (Fig. 13A). However, in the sections without immuno-staining of the primary anti-twitchin antibody, gold particles were not observed (Fig.13B). These results indicated a possibility that twitchin molecules exist on the thick filaments, as reported in *Mytilus* ABRM[14].

Discussion

The present physiological experiments demonstrated the similarity in the nature of potassium contracture between the BWM of spoon worms and different muscle types, i.e. vertebrate and invertebrate smooth muscles and frog skeletal muscles[20-22]. In these cases, the peak contracture tension was related to the log [K]o by an S-shaped curve (Fig. 3). In the BWM, however, the difference among the three types of muscles was reflected as a shift of the S-shaped curve in Fig. 3. This suggests that their response to [K]o is slightly different.

The development of K-contracture tension in all BWM fibers was repressed to a certain extent by the removal of external Ca (Fig.5), and in ICBWM fibers it was enhanced by the increase in [Ca]o (Fig. 3). Furthermore, in ICBWM fibers, K-contracture was repressed by Mn ions, La ions, and verapamil, which block Ca influx in excitable membranes[23], and vertebrate and invertebrate smooth muscles[6, 9, 22, 25]. These results indicate that activator Ca of BWM is partially supplied from the external solution.

On the other hand, in Ca-free ASW, caffeine induced contraction in BWM (Fig. 7). It is well known that caffeine causes contraction in various skeletal and smooth muscles as a result of Ca release from the SR[22, 25, 26]. In addition, ultrastructural observations and image analyses revealed that the BWM fibers contained a large number of vesicular SR forming diads with the plasma membrane (Fig. 9),

Table 2. Relative concentration ratios of elements in the pyroantimonate precipitate in the ICBWM fibers of spoon worms

Element-line	Relative concentration ratios		
	Resting fibers		Contracting fibers
	Plasma membrane (n=15)	Sarcoplasmic reticulum (n=15)	Myoplasm (n=30)
Sb-Lα	1.000	1.000	1.000
Ca-Kα	0.063 ± 0.025	0.061 ± 0.016	0.030 ± 0.015
K-Kα	0.016 ± 0.011	0.023 ± 0.013	0.040 ± 0.016
Mg-Kα	0.008 ± 0.006	0.012 ± 0.009	0.011 ± 0.004
Na-Kα	0.000	0.000	0.001 ± 0.004

Values are the mean±SD (n=12).

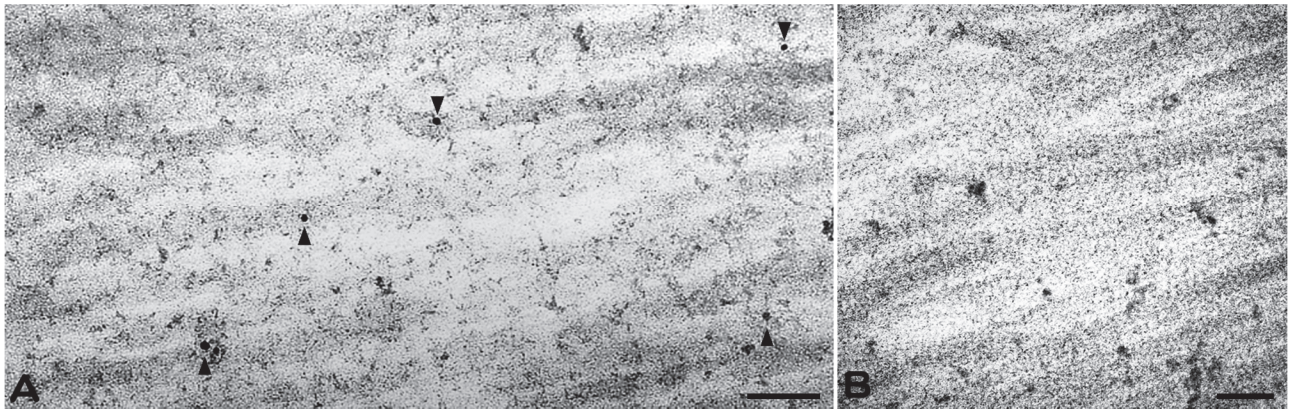

Fig. 13. Immuno-electron microscopy. A. Electron micrograph of a section immuno-stained with anti-twitchin antibody, showing antibody-conjugated gold particles (arrow heads) located on the surface of thick filaments. B. Electron micrograph of a section without the primary anti-twitchin antibody. Scale bars, 200 nm (A) and 100 nm (B).

and its fractional volume was relatively high (Table 1). Thus, the present results suggest that activator Ca is partially supplied from the SR.

When Ca and Mg ions were removed from the external solution, the BWM exhibited irregular or oscillatory contraction (Fig. 8). Such mechanical responses closely resemble those observed in invertebrate smooth muscles[4, 6, 17, 25], and may be caused by activator Ca released from the inner surface of the plasma membrane when it becomes unstable due to the lack of divalent cations. Thus, there may be the second intracellular Ca-accumulating structure in BWM, i.e. the inner surface of the plasma membrane.

Cytochemical experiments demonstrated that, in the BWM fibers, the PA precipitates were mostly localized in the SR and along the inner surface of the plasma membrane at the resting state, whereas, concomitantly with the disappearance of precipitates from these structures, they were diffusely distributed in the central region of the myoplasm at the contracting state. In cytochemical observations, no difference was found among the three types of BWM fibers. In addition, the X-ray microanalysis of PA precipitates observed in resting and contracting fibers revealed that they contain Ca. These results are consistent with those of previous studies on Ca localization and translocation during mechanical activity in vertebrate and invertebrate smooth muscles by the PA method[4-10]. Therefore, in BWM fibers, Ca accumulated in the SR and inner surface of the plasma membrane may be released into the myoplasm to cause contraction during mechanical activity. As demonstrated by the physiological experiments, activator Ca may also be supplied from the external solution via Ca influx.

Acknowledgments

The authors express their hearty thanks to Dr. Eigoro Tazawa and Professor Akiya Hino (Kanagawa University) for suggesting the use of body wall muscles of spoon worms for studies on the regulatory mechanism of Ca in smooth muscles.

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