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Contractile Units in Mammalian and Amphibian Visceral Smooth Muscle Cells

(myofilaments/contracted and relaxed stomach/calcium ions)

MITSURU OKI, OSAMU TANAKA, TOKIO DOMOTO, and TATSUO GONDA

Department of Anatomy, Shimane Medical University, Izumo 693, Japan

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Three types of myofilaments in the stomach muscle were studied with an electron microscope, in connection with isometric and isotonic contraction. In dogs, generally, thick and thin filaments were observed in the contracted muscles. In frogs, thick filaments were found only in the contracted muscles of which myoplasm invariably contained calcium (oxalate or pyroantimonate) deposits. In frog stomach, contracted muscles often contained lattice-like arrangements of thin filaments, suggesting molecular changes. Dense bodies were often contracted and associated with contraction of thin filaments. It was postulated that the dense bodies-thin filaments system is functionally equivalent to the sarcomere of the striated muscles. Both contraction-relaxation cycles and formation of thick filaments are probably regulated by the intracellular concentration of free calcium.

Contraction and relaxation of vertebrate smooth muscle, as in vertebrate striated muscle, have been studied on the basis of a sliding interaction of thick and thin filaments (1—10). In the vertebrate visceral smooth muscle, three types of myofilament have been identified by means of the electron microscopy (5—9) ; these include numerous thin filaments (actin, 4.8—8.0 nm in diameter), a much smaller amount of thick filaments (myosin, 13—18 nm in diameter) and intermediate-size filaments (mostly, 10 nm in diameter). A variety of each diameter in both thin and thick filaments may imply that smooth muscle filaments differ in forms in different organs and species (12). It has been reported that the thin filaments appear to be oriented parallel to the longitudinal axis of the cell in both contracted and relaxed muscles, and are poorly organized in bundles (1, 2, 8). The thick filaments are rarely visible in most of the smooth muscle fixed by usual procedures (2, 6) ; they are also rare or absent in the relaxed smooth muscle in which they appear to be present in an unaggregated form, while they aggregate into discrete filaments during contraction (2, 3, 6). It may be significant that the thick filaments were absent when muscles were preincubated in calcium-free solutions containing EDTA (5, 10). The intermediate filaments are labile and less numerous in number than the thick filaments ; they appear to be aggregated around the periphery of the intracellular dense bodies (5—9), which have been considered as attachment sites for the thin filaments similar to the Z-lines of the striated muscle (6). Thus, the intermediate filaments have been regarded

as cytoskeletal components (5, 7, 9, 11) ; and they appear not to be contractile in nature. Current investigations of vertebrated smooth muscles, including the structural and biochemical (1, 2, 14) and the mechanical problems (13), are summarized in recent reviews. Ultrastructural analysis regarding myofilament-contraction has yet to be made.

In this study, the gastric smooth muscles were examined in connection with physiological and ultrastructural observations. Comparative data concerning the contractile apparatus in the canine and the frog stomach muscles are also discussed.

MATERIALS AND METHODS

Physiological Experiments

Preparations : Toads (*Bufo vulgaris japonicus*) in spring and bullfrogs in summer were used. The whole stomach was excised from anesthetized frogs and transferred into a bath containing a standard Ringer solution. After both mucosa and serosa were peeled off, muscle strips 15 mm long and 4 mm wide were excised from the middle portion of the stomach ; and were perfused in Ringer solution bubbled with 5% CO_2 in oxygen and kept at 22 to 28°C.

Solution and Fixatives : (1) Amphibian standard Ringer solution contained (mM) : NaCl 112, KCl 2.0, $CaCl_2$ 1.1, and Na-phosphate buffer to give pH 7.1. Calcium-free Ringer solution contained 0.2 mM EDTA. Isotonic sucrose solution containing 2 mM $CaCl_2$ was also used as a washing solution. All incubation media were adjusted to pH 7.1.
(2) Mammalian physiological solution (Tyrode) contained (mM) : NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.33 and glucose 11.2.
(3) Fixatives used were 3% glutaraldehyde and 1.5% osmium tetroxide made up in whichever solution or in the phosphate buffer.

Mechanograms : In an attempt to make fixations of muscles, either at the peak of contraction or during the periods of complete relaxation, the mechanical activities of the muscles were recorded. Isometric contraction curves were recorded by means of a method previously described in detail (15).

Muscle strips of frog stomach were often quiescent in Ringer solution when not stimulated. To record contractures, individual strips were exposed to an 30—115 mM potassium Ringer solution, in which sodium ion was replaced by potassium ion, on a molar basis. In an attempt to induce relaxation, the muscle strips were incubated in Ca-free Ringer solution for up to 120 min. Before experiments, the muscle strips were equilibrated in the bathing solution for 60 min, and the solution was changed at 10-min intervals.

To induce muscle-contractures of the dog stomach an injection of DMPP (1, 1-dimethyl-4-phenylpiperazium iodide) was given into the right gastroepiploic artery at total concentrations varying between 0.5 and 2.0 mg.

Electron Microscopy

To examine the localization of calcium ions in smooth muscles, some of the frog muscle strips were immersed in an individual fixative containing either 2% potassium pyroantimonate or 0.25 mM ammonium oxalate. Other technical details were as described in previous papers (15, 16).

RESULTS

Typical patterns of the potassium-induced contracture, such as those shown in Fig. 1a, were invariably recorded when the circular muscle strips were

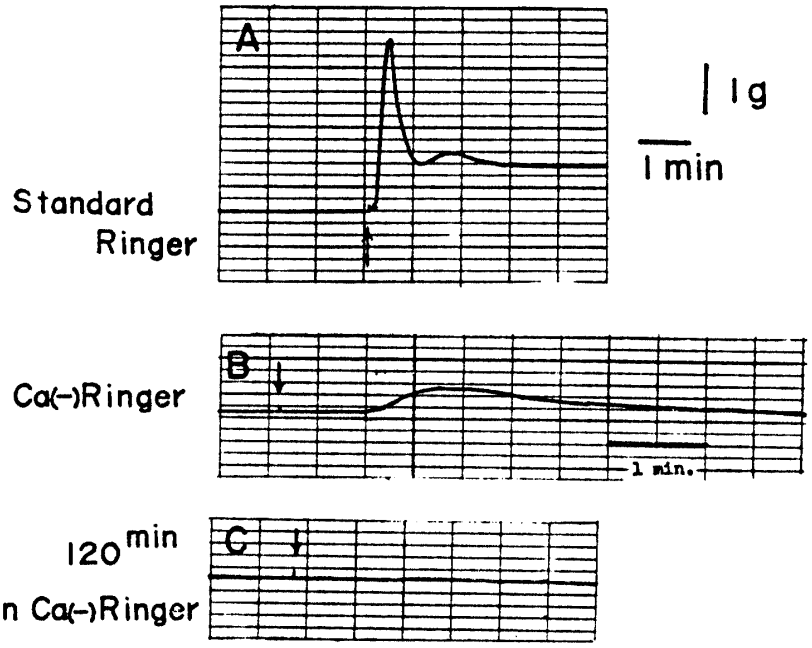
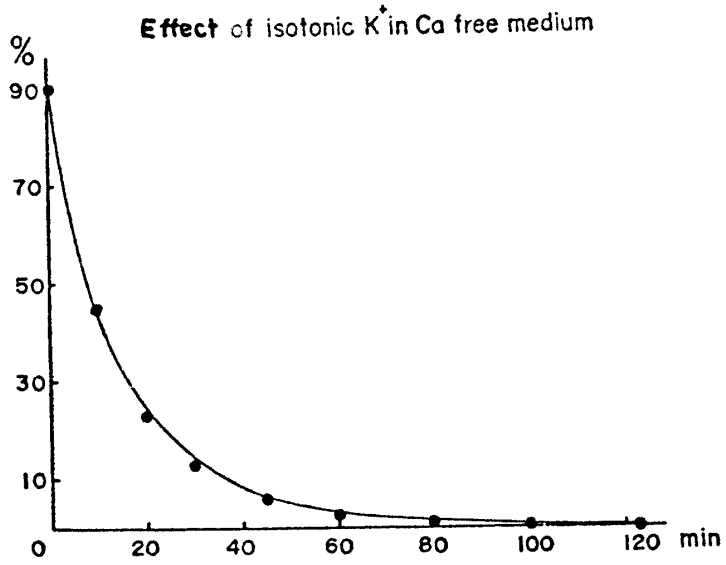


Fig. 1a. Isometric responses of muscle strips (toad stomach) to isotonic potassium Ringer and effect of Ca-deprivation ; A, first record (control) of K^+-induced contracture during perfusion with standard Ringer ; B, subsequent record (40 min after record of A) during perfusion with Ca-free solution ; C, 120 min after record of A, showing disappearance of tension owing to Ca-deprivation. In A-C, arrow shows replacement of standard Ringer by K^+-Ringer, which was applied for 3 min.

exposed to an isotonic KCl-Ringer solution ; the patterns consisted of an initial rapid and a second long-lasting tonic component. The initial rapid component was phasic in nature and served as a control tension. After recordings of the first contracture, the muscle was immersed in Ca-free Ringer solution ; this solution was renewed every five min throughout the experiment ; then, in the subsequent 40 min in Ca-free Ringer solution, the potassium-induced contracture showed remarkable reductions (Fig. 1a-B) ; particularly, the amplitude of the phasic contraction was markedly diminished. When the immersion in Ca-free solution was continued for more than 120 min, the muscle invariably failed to respond to the isotonic K^+-solution (Fig. 1a-C) ; however, the contracture by potassium ion was fully restored on returning

the muscles to standard Ringer solution. Similar results were also confirmed in many experiments. Typical results are shown in Fig. 1b in which the



Fib. 1b. Relationship between K^+-induced tension and time. Ordinate : magnitudes of K^+-induced maximum tension shown as a percentage of that in control. Abscissa : time elapsed in Ca free Ringer. In Figure, the points represent means of three individual tensions. (The limits of S. D. are neglected). Successive records of maximum tension in Ca free Ringer showed their progressive decline and disappearance after 120 min.

potassium-contracture was repeatedly induced at about ten-min intervals ; the curve indicates a decline of the amplitude of phasic contractions, depending on the elapse of time in Ca-free Ringer solution. It should be noted that about 50% of the initial tension was reduced during the first 10 min in Ca-free Ringer solution and 90% within 40 min, and that subsequent reduction was slow. In this and many other experiments, the time taken to abolish both the phasic and tonic components varied from 110 to 140 min, and seemed dependent on the manner of changing bathing solutions. The present results are supported by the findings of Bozler (17), who by chemical analysis found that in Ca-free Ringer solution, frog stomach muscles lose about half of their calcium in 10 min and exchangeable total Ca^{++} in 2 hr. It has been also reported that in Ca-free Ringer solution, frog stomach muscles become unresponsive, usually after several hours (18). Thus, it is likely that calcium is essential for the phasic and the tonic contraction of the frog stomach muscle.

The nature and mechanism of these responses, however, will be the subject of another paper (19—21). The purpose of the present experiments, as stated in the introduction, was to fix the muscles in either the fully relaxed or contracted state. Thus, changes in the tension of the smooth muscle strips could be correlated with those in the ultrastructural organization of myofilaments.

Electron Microscopy

Relaxed Muscle (Frog stomach)

Relaxed muscles were prepared by immersing them either in Ca-free Ringer

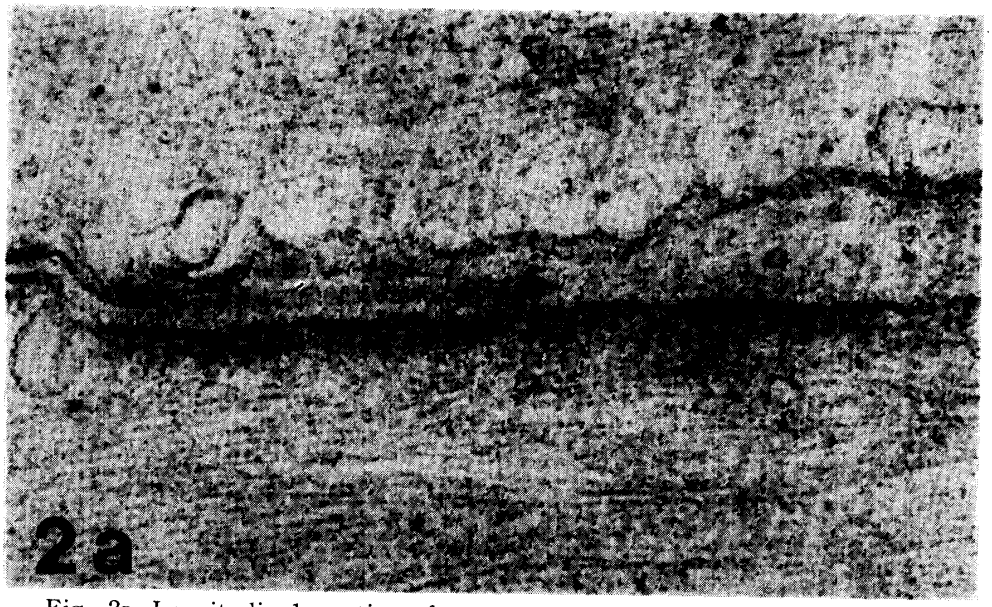


Fig. 2a. Longitudinal section of two muscle cells relaxed with 10^{-7} g/ml adrenaline in Ringer solution. Loosely packed and longitudinally oriented thin filaments only are seen. There are also filamentous plaques beneath the plasma membrane into which thin filaments insert. (toad stomach) ×100,000

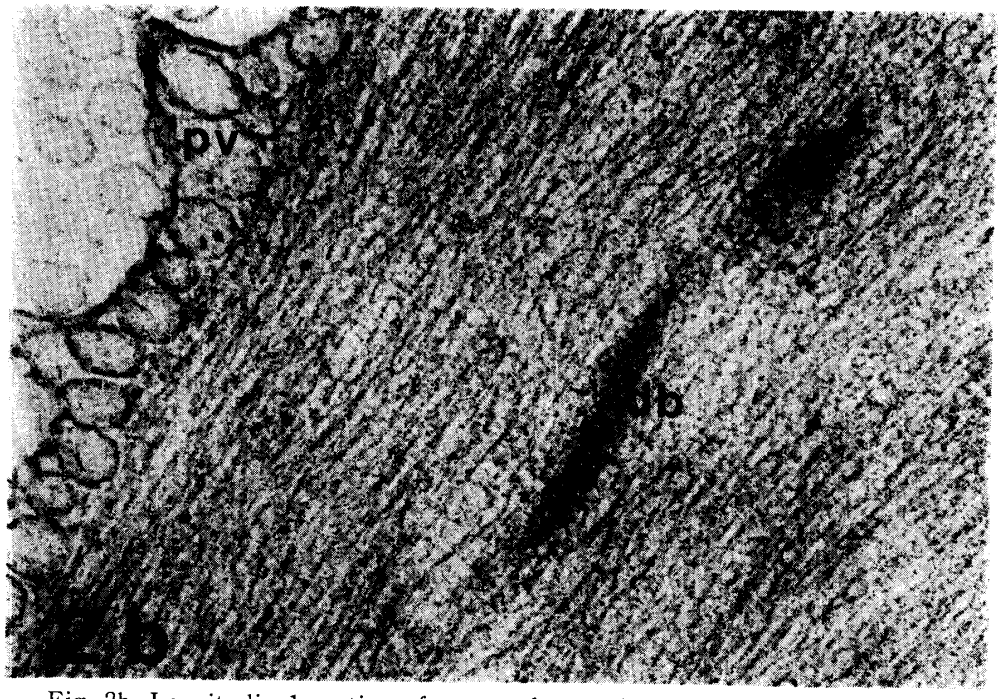


Fig. 2b. Longitudinal section of a smooth muscle cell relaxed in Ca-free Ringer, containing 0.2 mM EDTA. Only thin filaments parallel to the cell axis and less dense bodies (db) are present. Pinocytotic vesicles (pv) are free of myofilaments. (toad stomach) ×75,000

(Fig. 2b) or in standard Ringer solution containing adrenaline (10^{-7} g/ml in Fig. 2a) ; and four typical changes were observed. These included : smooth contours of the plasmalemma ; decreased numbers of plasmalemmal vesicles ; loosely packed and more or less longitudinally oriented myofilaments ; and a much smaller amount of or lack of cytoplasmic dense bodies. The cytoplasm contained only thin filaments approximately 4.5 to 6.5 nm in diameter. The densely stained filamentous plaques, which have been considered to be the anchorage points for the thin filaments (6, 8, 12), were often prominent beneath the plasma membrane (Fig. 2a). Often small, prickle-like cellular projections and increased numbers of plasmalemmal vesicles were observed in the calcium-deprived smooth muscles (Fig. 2b). These structural changes were usually reversible on returning the muscle to standard Ringer solution. It is also noteworthy that thin filaments as well as other types of filaments were rarely demonstrated when the muscle was perfused with Ca-free Ringer solution containing EDTA (1 mM) for up to two hours.

Contracted Muscle (Frog stomach)

Isometric contraction was induced by application of excess K^+-Ringer solution ; contracted muscle cells were still fusiform in shape and in more or less parallel alignments. Prominent changes were as follows ; there was an increase in the number of thin filaments. Thicker filaments, ranging 10 to 13 nm in diameter, were also seen but only in the contracted muscles. In this context, thicker filaments, as discussed in detail later, are probably

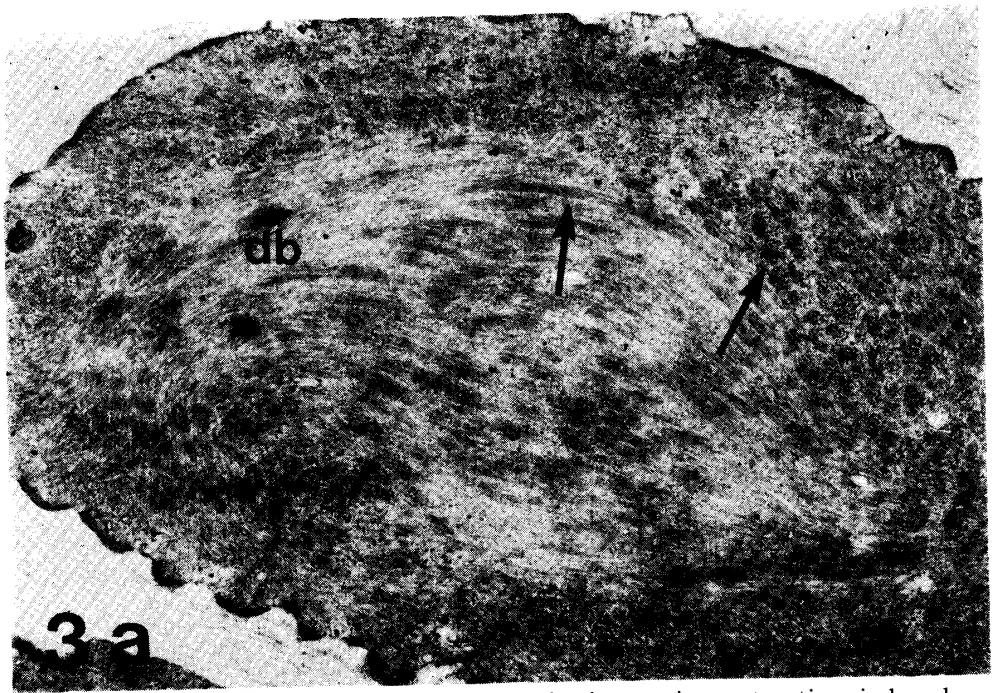


Fig. 3a. Cross-section of a muscle cell in isometric contraction induced by potassium-depolarization. Contracted muscle cell exhibits irregular contours of plasmalemma, and contains variable orientation of thin filaments (arrows) and increased numbers of dense bodies(db). (bullfrog stomach) ×24,000

Fig. 3b. Longitudinal section of thin filaments organized in whorls, indicating contraction in their concentrical arrangements. Some of the thin filaments penetrate dense bodies (db). Calcium oxalate-deposits are seen in the cytoplasm and are detected only in contracted muscle cells. Figures 3a and 3b were obtained from the same specimens. ×62,500

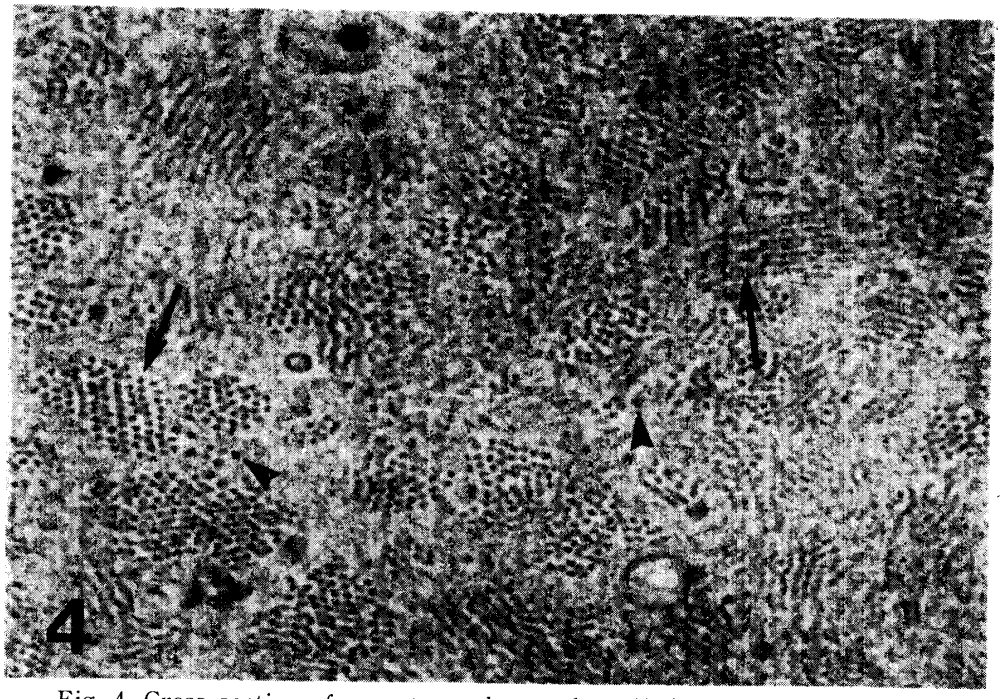


Fig. 4. Cross section of a contracted muscle cell, in which myofilaments show variable orientation ; some of the thin filaments are in longitudinal or oblique and others in cross sections, the later of which is arranged in hexagonal or polygonal lattice. (Both are shown by arrow). Arrowhead shows thick filaments. (potassium-induced contracture of bullfrog stomach muscle) ×120,000

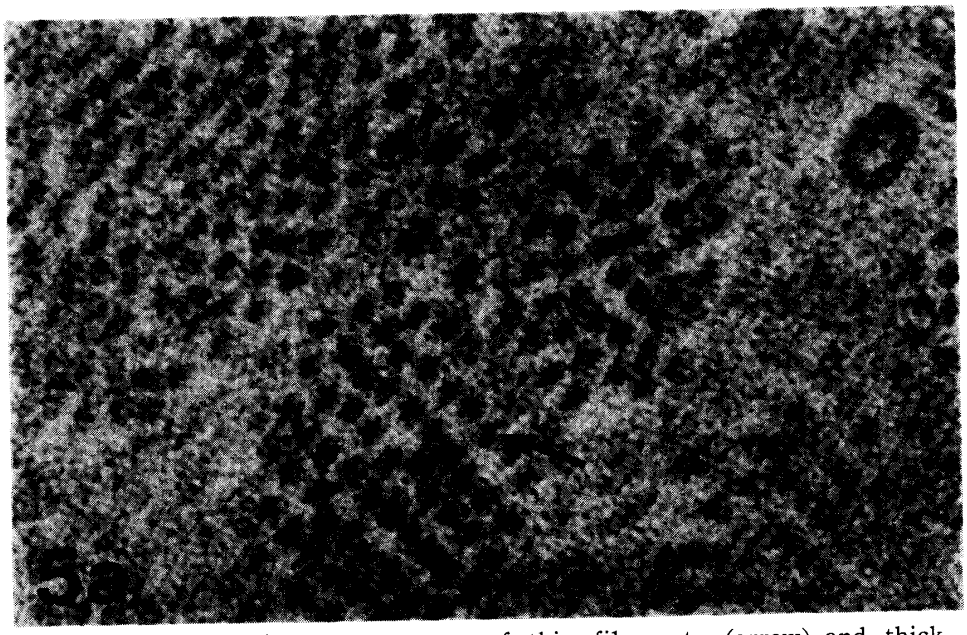


Fig. 5a. Lattice-like arrangements of thin filaments (arrow) and thick filaments (arrowhead). Thin filaments contain one or more grains, suggesting their molecular arrangements. There were few thick filaments (arrowhead). ×360,000

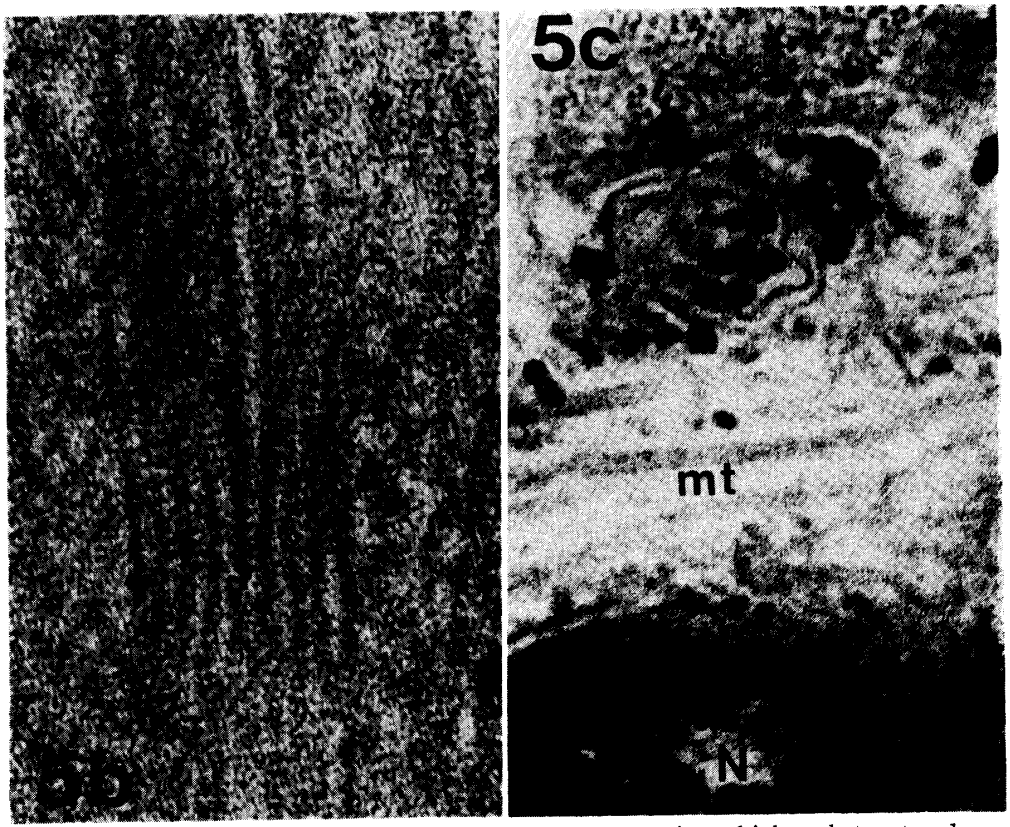


Fig. 5b. Longitudinal section of thin filaments, in which substructural grains are not identified. (bullfrog stomach) ×284,000

Fig. 5c. Microtubles, ranging from 20 to 30 nm in diameter, in the longitudinal section ; microtubles are more frequently observed in the contracted muscle. Calcium deposits are also seen mainly in the endoplasmic reticulum. mt, microtubles ; N, nucleus (bullfrog stomach) ×124,000

identical with the conventional thick filaments. The plasmalemma of muscles exhibited small irregular contours with increased numbers of pinocytotic vesicles where there were no myofilaments. The cytoplasm contained increased numbers of dense bodies and appeared more electron dense than that in control muscles (Figs. 3a to 5c). It must be emphasized that many cells fixed in contraction contained only thin filaments in many sections. Figs. 3a and 3b show cross-sectioned profiles of a contracted muscle in which the longitudinally sectioned thin filaments are seen to be in circular orientation around the central area of the cell, while in other areas the thin filaments are found in transverse or oblique sections ; the longitudinally sectioned thin filaments appear to be organized in discrete bundles. Profiles of these bundles exhibit both concentrical and lattice-like arrangements (Figs. 3a, 3b and 4). As can be seen in Fig. 5a, the lattice-like arrangements of myofilaments consist of mainly thin filaments among which there are also thicker filaments. It should be emphasized that thin filaments had one or more grain-like cores inside ; the core could not be identified in the longitudinal section of the filaments (Fig. 5b). Occasional microtubles, ranging from 20 to 30 nm in diameter, were also observed in the contracted muscles (Fig. 5c).

Contracted Dog Stomach

Isotonic contraction was induced, *in situ*, either by applying DMPP (in Figs. 6a and 6b), or by stimulating the vagus nerve, Nervi of Latarjet, (in Fig. 7) respectively ; during contraction, the whole stomach was fixed by means of arterial perfusion with the fixatives, then excised and used for electron microscopy.

Fig. 6a. Contraction of dense bodies in an oblique section. Undulation of dense bodies (db) is consistently related to muscle contraction. (dog stomach in isotonic contraction) ×50,000

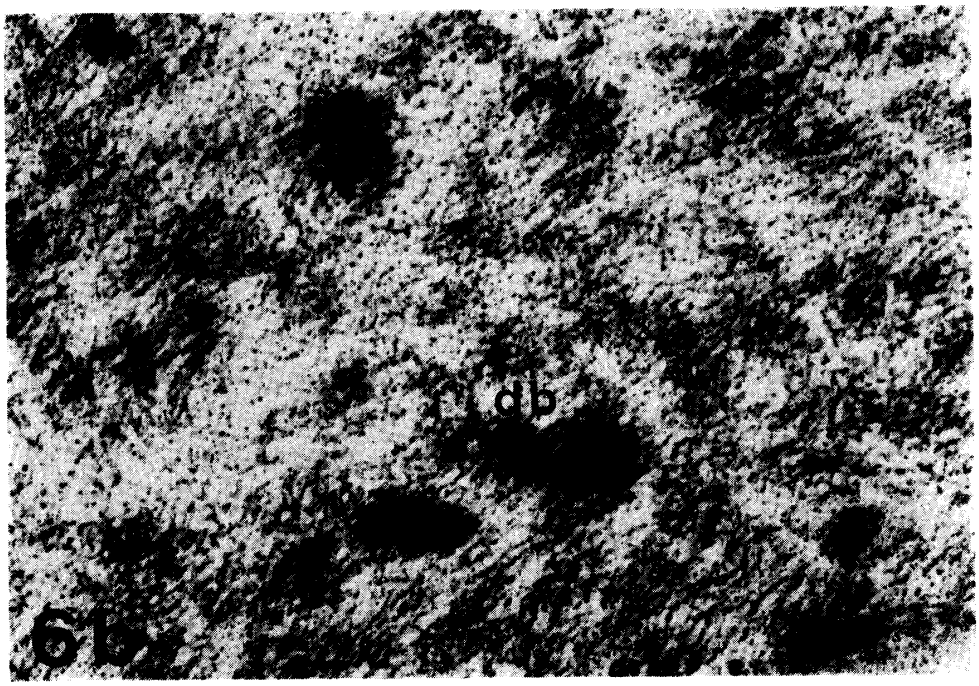


Fig. 6b. Dense bodies in the isotonically contracted muscle ; they appear to be attachment sites of thin filaments. (dog stomach) ×77,500

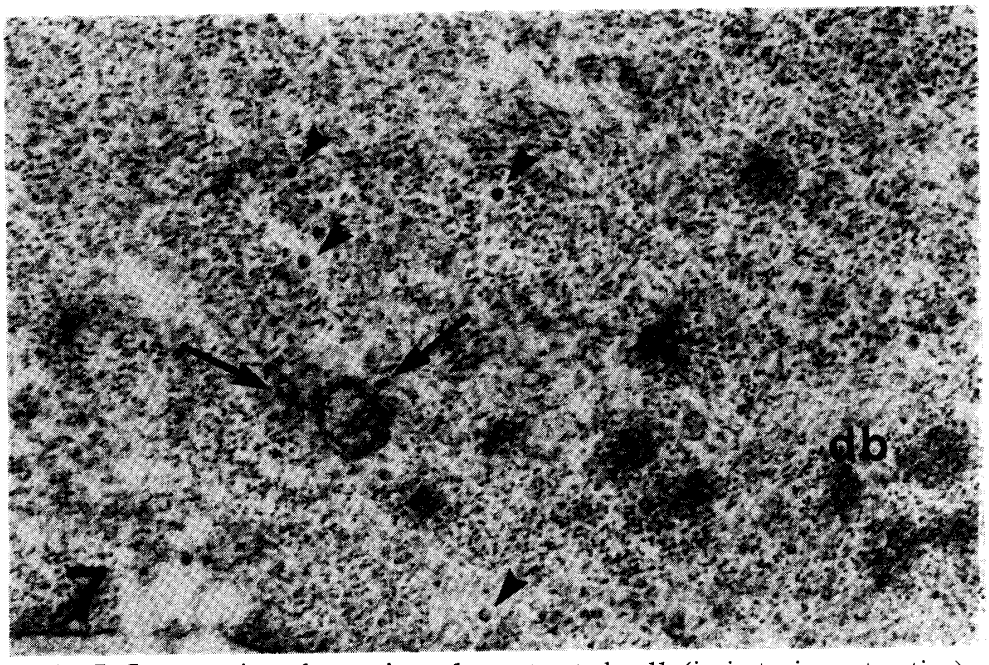


Fig. 7. Cross section of a moderately contracted cell (in isotonic contraction). Intermediate filaments appear to be aggregated around or inside of dense bodies (db). Microtubles (arrowhead) appear to be surrounded by a halo. arrow, thick filaments (dog stomach) ×45,500

These contracted muscles also contained three types of myofilaments and measured, in diameter, approximately 15 to 18 nm, 10 nm and to 8 nm respectively. Of these, intermediate filaments often aggregated around the periphery of the cytoplasmic dense bodies into which thin filaments also appeared to have penetrated (Figs. 6b, 7). Thus, the intermediate filaments

and dense bodies-complex may be identical (5, 7, 9, 11) with an intracellular cytoskeleton. It is noteworthy that in contracted muscle, the number of dense bodies increased and in some, contractions often occurred (Fig. 6a). Thick filaments were more prominent in contracted muscles and increased in number. Other profiles of the contracted muscles in canine stomach were characterized by highly irregular contours of their plasmalemma with numerous pinocytotic vesicles, increased numbers of protrusions and invaginations, different orientations of myofilaments, increased densities of cytoplasm, convoluted nucleus and numerous mitochondria. These changes were much more prominent in the isotonically contracted muscle thanin the isometrically contracted muscle.

Calcium deposits, calcium oxalate or calcium pyroantimonate, were invariably found in the myoplasm of the contracted smooth muscles (Figs. 3a and 3b). If the muscles were immersed in Ca-free solution (containing 0.2 mM EDTA) for more than 2 hr, the muscles eventually failed to contract by potassium-stimulation and lost calcium-deposits in their myoplasm. The localization of calcium ions in the gastric musculature will be described in detail in a subsequent paper.

DISCUSSION

The present results show that the contracted smooth muscles in frog stomach contained thin filaments (5 to 7 nm in diameter) and thicker filaments (10 to 13 nm in diameter), whereas the relaxed muscles had only thin filaments ranging from 4.5 to 6.5 nm in diameter. There have been two studies on the contracted muscle in frog stomach (22, 23) in which identification of the myofilaments was not made and the class of filaments not confirmed. In electron microscopy, it has been reported that preservation of amphibian smooth muscle is invariably more difficult than that of mammalian smooth muscles (24). Satisfactory preservations of the thick filaments may be related to several factors : e. g. poor fixation including cell swelling, pH in medium (alkaline pH is not adequate), temperature and so on (25, 26). It is interesting to note that in the present study, the thicker filaments in frog stomach were identifiable only in the transverse section of the contracted muscle, but not in the relaxed muscle. Consequently, their longitudinal organization as well as their length are quite unknown. Assuming different orientations of variable sections, the thicker filaments might be regarded as minute, rod-like filaments. In this respect, Somlyo and Somlyo observed cylindrical filaments in freeze-fractured preparations, thus providing substantial evidence for their form (27). In guinea pig taenia coli (27) and rabbit mesenteric vein (9), thick filaments are 15 to 18 nm in diameter and can be seen to taper at the ends (28). It is now generally accepted that myosin is organized into thick filaments in vertebrate smooth muscle, and actin into thin filaments. From the above evidence and based on a two-filament sliding hypothesis in muscle contraction, the thicker filaments observed in the present study are

assumed to be identical with the conventional thick filaments (15 to 18 nm-filaments). However, the amount of the thicker filaments was so little even in the contracted muscles, and appeared insufficient to explain the two filaments interactions. In this respect, there is convincing evidence in favour of the present observation ; thick filaments, are highly labile in the absence of calcium ion as these filaments are not present in homogenates when Ca ions are chelated by EGTA (30, 31). It is therefore suggested that thick filaments might only occur *in vivo* in the presence of calcium ion, i. e. during contraction (30). Thus, some investigators believe that most of the thick filaments, but not all, exist in a depolymerized state in the relaxed muscles and that they aggregate into discrete filaments with excitation-contraction coupling (29) (also see review by Shoenberg and Needham (12)). From the serial electron microscopy of depolarized muscles as induced by potassium ions, we postulate that in frog stomach muscles both contraction-relaxation cycles and formation of thick (myosin) filaments are regulated by the intracellular concentration of free calcium. This is also supported by the fact that the presence of intracellular Ca^{++}-deposits was invariably associated with the existence of thicker filaments. In the present study, we used Ca^{++}-free solution containing a small amount of EDTA (0.2 mM). Fay and Cooke (5, 11) found that the presence of EDTA (5 mM) in Krebs solution caused disruptions of both thin and thick filaments. Consequently, it may be necessary to examine the effect of EDTA on the preservations of both myofilaments.

Heuman (32) observed lattice-like arrangements of the thin filaments, particularly in the relaxed smooth muscle. However, we found such structures only in the contracted muscles after stimulation with K^{+}-ions. As is evident from our electron micrographs (Figs. 3b and 4), the lattice-arrangements are equivalent to the contracted thin filaments regularly ordered into bundles. We also point out that small portions of these arrangements were rarely observed in the control materials. It is also noted that the filaments in lattice-arrangements contain one or more grain-like cores (approximately 1.5 to 2 nm in size) suggesting the presence of substructural elements.

Ultrastructurally, F-actin filaments which are identical with the thin filaments suggest that they consist of double helices made up from chains of G-actin sub-units ; the strands cross over every 36−37 nm along the actin filament ; G-actin in electron microscopy is a sphere of about 5.5 nm in diameter (see Text by Huddart and Hunt (6)). Considering the molecular compositions of the thin (actin) filament, explanation for the grain-like cores remains unknown. Further observations are necessary to determine the location of the tropomyosin molecules. Some investigators reported lattice-like arrangements of the thick filaments (28, 29). However, we found no evidence of such arrangements. Intermediate filaments sometimes contained a less electron-opaque central core and appeared to aggregate around dense bodies. These filaments appear to serve as cytoskeleton (5, 7, 9, 11) and are not contractile in nature (11). Their major protein component has a molecu-

lar weight of 55,000 (14) and has been termed skeletin (9). These authors (9, 11) also mentioned that intermediate filaments penetrate into the inside of dense bodies ; ultrastructually Somlyo and Somlyo (27) claimed that they were not in connection with dense bodies. There is disagreement among investigators concerning the functions of the dense bodies ; Small (9) and Cooke (11) found no evidence for the presence of thin filaments linking up with dense bodies, whereas Somlyo and Somlyo (27) and Ashton *et al.* (28) did ; the latter being in agreement with our evidence. In addition, we have observed the contracted profiles of dense bodies. Consequently, it is postulated that the dense bodies-thin filaments system is functionally equivalent to the sarcomere of the striated muscle. Based on the present results, additional conclusions are that in isotonic but not isometric contractions, the muscle cells can largely change their forms, and that peculiar structural changes of the myofilaments occur.

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