THICK FILAMENTS IN VASCULAR SMOOTH MUSCLE

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ABSTRACT

Two sets of myofilaments were demonstrated after incubation of strips of rabbit portal-anterior mesenteric vein under moderate stretch in a physiological salt solution. Thick filaments had a mean diameter of 18 nm and reached a maximum length of 1.4 μm with a mean length of 0.61 μm. In transverse sections, 2.5–5 nm particles were resolved as subunits of the thick filaments. Thin filaments had an average diameter of 8.4 nm and generally conformed to the structure believed to represent actin filaments in smooth and striated muscles. In the areas of maximum concentration there were 160–328 thick filaments/μm^2 and the lowest ratio of thin to thick filaments was 12:1. Thick filaments were present in approximately equal numbers in vascular smooth muscle relaxed by theophylline, in Ca^{++}-free solution, or contracted by norepinephrine. The same preparatory procedures used with vascular smooth muscle also enabled us to visualize thick filaments in guinea pig and rabbit taenia coli and vas deferens.

Thick filaments in vertebrate smooth muscle have been observed in only a relatively few studies (Choi, 1962; Needham and Shoenberg, 1964; Fawcett, 1966; Nonomura, 1968; Kelly and Rice, 1968; 1969; Yamauchi and Burnstock, 1969; for reviews see Somlyo and Somlyo, 1968; 1970), and the reasons for the inconsistent visualization of thick myofilaments (2–5 times the width of thin filaments in the same preparations and ranging approximately 12–24 nm in recent studies) in these preparations are little understood. Thin filaments (ranging from 3 to 10 nm in diameter) are generally present in smooth muscle (Hanson and Lowy, 1963; Needham and Shoenberg, 1967; Rice et al., 1970), and some authors claim these to be the only filament type present (Panner and Honig, 1967; 1970).

The following explanations have been suggested for the variable presence of thick myofilaments in mammalian smooth muscle: (*a*) thick filaments are normally present but are destroyed during preparation of the tissue, perhaps because of the marked solubility of the constituent myosin, even in media of low ionic strength. Thick filaments can be demonstrated when smooth muscle is prepared by inert dehydration for electron microscopy instead of chemical fixation (Pease, 1968), but the disadvantage of inert dehydration is that it does not allow visualization of thin filaments. (*b*) Thick myofilaments are absent in relaxed smooth muscle but myosin is organized into thick filaments at the moment of excitation-contraction coupling; hence, thick filaments can be demonstrated only in contracted muscle (Shoenberg, 1969; Kelly and Rice, 1969). (*c*) Myosin is not organized into thick filaments, and the thick filaments, when visualized, represent a preparatory artifact due to the coalescence of thin myofilaments (Panner and Honig, 1970). This explanation also includes the possibility that myosin filaments are sufficiently similar in size to actin

filaments to be indistinguishable in routine electron microscopy.

The present studies resulted from the chance observation of large numbers of thick filaments in vascular smooth muscle of the rabbit investigated during a survey of the sarcoplasmic reticulum (Devine and Somlyo, 1970). In many cases, we found thick filaments in sufficient numbers to allow quantitative estimation of their size and distribution within the smooth muscle cell. Thin filaments were also abundant, permitting us to estimate the relationship between the two sets of filaments. We also explored the effects of the contractile state and the presence of calcium on the visualization of thick myofilaments.

METHODS

Initially, vascular smooth muscle cells incubated with horseradish peroxidase in vitro (Cotran and Karnovsky, 1968) were found to have large numbers of thick filaments, 15–28 nm in diameter (reported in Somlyo and Somlyo, 1970). The effects of incubation, stretch, and drug treatment on the ultrastructure of the myofilaments in vascular smooth muscle and other smooth muscle were then investigated without the addition of exogenous peroxidase.

All tissues were obtained from rabbits and guinea pigs killed by cervical dislocation or decapitation.

Drug Treatment and Contractile State

The presence of thick filaments in various contractile states of the smooth muscle was investigated in longitudinal strips of the rabbit portal-anterior mesenteric vein. Several prefixation procedures were followed, including stretch to approximately 1.5 times the excised length, and incubation in Krebs' bicarbonate of the following composition (mM): NaCl, 118.9; KCl, 4.7; $CaCl_2$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; Glucose, 5.6. The solution was bubbled with 95% O_2–5% CO_2. L-norepinephrine bitartrate (10 μg/ml, Sigma Chemical Co., St. Louis, Mo.) was used to contract vascular smooth muscle during the 5 min preceding fixation. Theophylline (2.5 mM, Nutritional Biochemicals Corporation, Cleveland, Ohio) was added as a vasodilator to maintain the vascular smooth muscle in the relaxed state. The calcium-free (Ca^{++}-free) medium did not contain added calcium, but no steps were taken to remove any calcium ions invariably present as impurities. Nonstretched preparations incubated in Krebs' bicarbonate bubbled with 95% O_2–5% CO_2, and stretched preparations not incubated (fixed immediately upon removal from

TABLE I

Effect of Stretch (1.5 times the excised length), Incubation, and Drug Treatment on the Presence of Thick Myofilaments after 2% Glutaraldehyde—2% Formaldehyde Fixation of Rabbit Blood Vessels

Vessel	Stretch	Total incubation time	Drug	Incubation time with drug	Thick myofilaments
		min		*min*	
mes. art.	–	30	–	–	++
mes. vein	–	–	–	–	+
mes. vein	yes	–	–	–	++
mes. vein	–	30	–	–	++
mes. vein	yes	10	–	–	+++
mes. vein	yes	30	–	–	+++
mes. vein	yes	30	NE	5	+++
mes. vein	yes	30	Theop.	5	+++
mes. vein	yes	30	Ca^{++}-free	30	+++
mes. vein	yes	30	CO_2	30	+++

A semiquantitative scale of +, ++, and +++ is used to designate concentrations of thick filaments found. + indicates a scarcity of thick filaments, ++ indicates that thick filaments are readily found but not in large numbers, and +++ indicates that thick filaments are found in approximately 50% or more of the smooth muscle cells. NE, norepinephrine 10 μg/ml; Theop., theophylline 2.5 mM; Ca^{++}-free, Krebs' bicarbonate without added calcium; mes. art., mesenteric artery; mes. vein, mesenteric vein. The tissues were incubated in Krebs' bicarbonate buffer bubbled with 95% O_2–5% CO_2, except for 100% CO_2 .

the animal) were also included (see Table I). In one instance, a rabbit portal-anterior mesenteric vein was fixed in vivo by dropping the fixative on the tissue. After the preincubation treatments, the strips of portal-anterior mesenteric veins and small mesenteric arteries were fixed in 2% glutaraldehyde–2% formaldehyde (prepared by heating paraformaldehyde powder in the buffer until dissolved) in 0.1 M sodium cacodylate buffer at pH 7.4 (G/F fixation). Some tissues were fixed in 2% glutaraldehyde with 12.6% sucrose in 0.1 M cacodylate buffer or in 4% glutaraldehyde in cacodylate buffer.

Investigation of Myofilaments in Different Tissues

Portions of small mesenteric artery (approximately 0.5 mm outside diameter), main pulmonary artery, taenia coli and vas deferens of rabbits, and the uterus (nonpregnant and pregnant), taenia coli, vas deferens and portal-anterior mesenteric vein of guinea pigs were stretched to 1.5 times of their resting length, incubated for 30 min in Krebs' bicarbonate bubbled with 95% O_2–5% CO_2, and fixed for 2 hr in G/F fixative.

All tissues were postfixed for 2 hr in 2% osmium tetroxide in 0.05 M cacodylate buffer pH 7.4. All tissues were stained for 30 min before dehydration in saturated aqueous uranyl acetate in distilled water, dehydrated in alcohol, and embedded in either Epon 812, Epon-Araldite 502 mixture, or Spurr low viscosity resin (Spurr, 1969). Sections were cut on a Porter-Blum MT 2B ultramicrotome, stained with alkaline lead citrate (Fahmy, 1967), and viewed in a Hitachi HU 11E electron microscope. The section thickness, estimated by interference colors (Peachey, 1958; Williams and Meek, 1966), was approximately 60–100 nm (silver-gold) for low power studies and general viewing, but was approximately 40 nm (grey) for high resolution studies. Both diamond and glass knives were used for sectioning.

The primary magnification used for high resolution studies was × 51,000. Filament dimensions were measured to the nearest 0.1 mm, with a × 8 eyepiece with a reticule, on micrographs enlarged optically to × 153,000 or × 306,000. The means were obtained graphically by the probability paper method (Harding, 1949; Cassie, 1950).

RESULTS

General Fixation

The smooth muscle cells were usually well fixed; the mitochondria and the sarcoplasmic reticulum were not swollen and the cell membrane was not pulled away from the cytoplasm (Figs. 1, 2). After prolonged incubation, the sarcoplasmic reticulum was sometimes swollen and the cell membrane was separated from the cytoplasm even though thick and thin myofilaments were present. This led to the general impression of bad fixation, but thick and thin myofilaments were found in tissues in which the over-all fixation was excellent (Figs. 1, 2).

Presence of Thick Myofilaments

The thick filaments (approximately 18 nm in diameter) were densely stained structures compared with the thin myofilaments (approximately 8 nm in diameter) found in almost all types of smooth muscle studied. Thick filaments were present in rabbit portal-anterior mesenteric vein (15 out of 18 embeddings), main pulmonary artery (2 embeddings), vas deferens (1 embedding) (Fig. 13), and taenia coli (2 embeddings) (Fig. 14), and in guinea pig taenia coli (3 of 4 embeddings), portal-anterior mesenteric vein (1 out of 3 embeddings, and vas deferens (1 embedding) after periods of stretch and incubation in the physiological medium. No unequivocal thick filaments were seen in guinea pig uterus (3 embeddings). There was clearly a fair amount of variability in the visualization of thick filaments among different embeddings, even in the absence of any known deviation from the routine preparatory procedures described. The effects of the different pretreatments on visualization of thick filaments were determined in the rabbit portal-anterior mesenteric vein and were generally graded upon examination of sections obtained from the same blood vessel fixed and embedded on the same date. The thick filaments seemed to be best preserved in the portal-anterior mesenteric veins fixed in 2% glutaraldehyde with 12.6% sucrose, but our material is insufficient for any quantitative evaluation of this finding. (See note added in proof).

The maximum concentration of thick filaments was 80–160 per μm^2 in transverse sections of mesenteric veins from two animals (including treatments with Ca^{++}-free solution, theophylline, and norepinephrine).[1] It was difficult to make a

[1] In selected mitochondrion-free areas with highly organized filament populations (e.g. glutaraldehyde with sucrose fixation, center section Fig. 8), thick filament counts up to 328/μm^2 were present. The extent of tissue shrinkage (hence change in filament

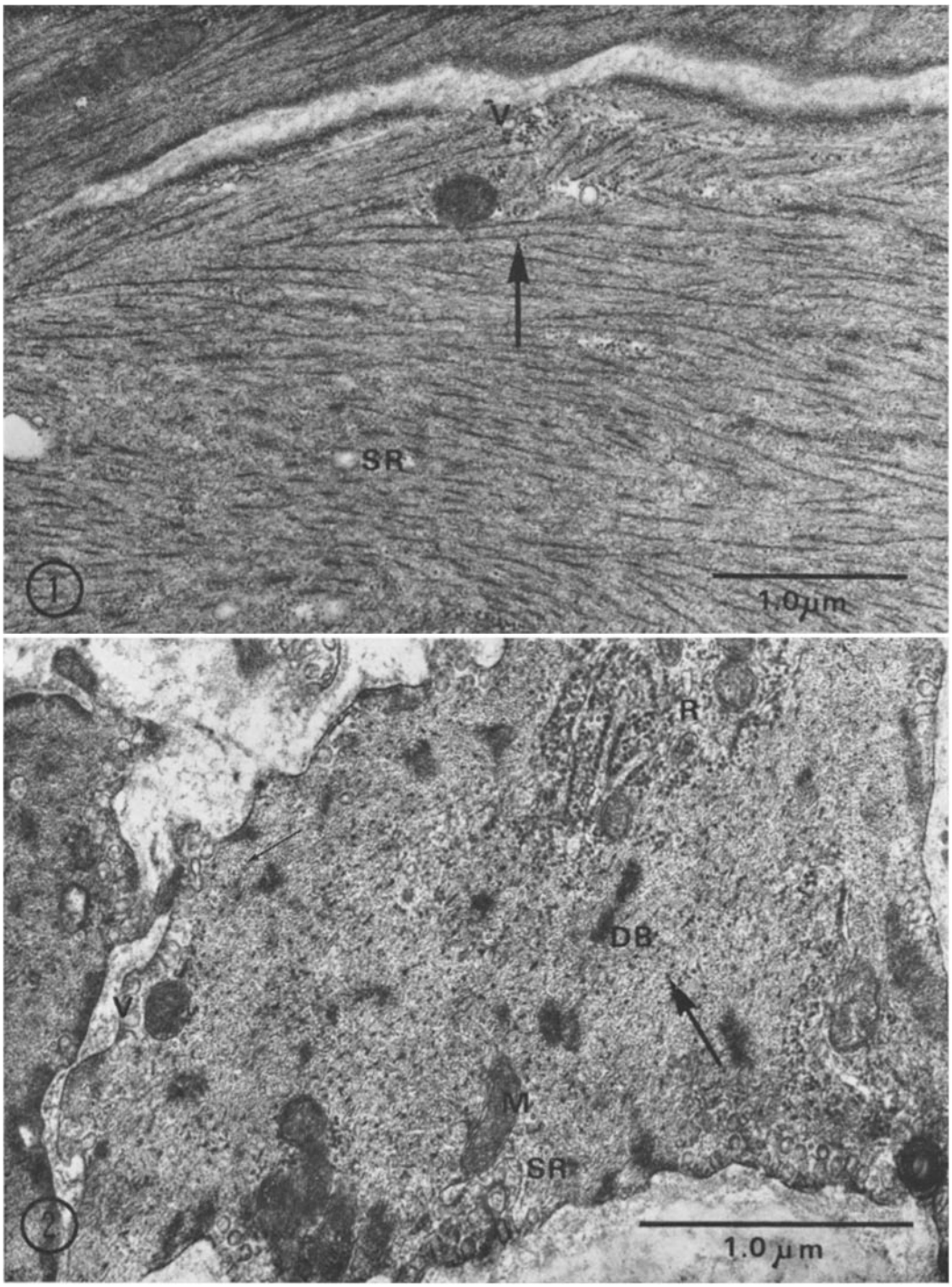


FIGURE 1 Longitudinal section through a vascular smooth muscle cell. Thick myofilaments (thick arrow) are oriented generally in a longitudinal direction, although obliquely-running myofilaments are also seen. Surface vesicles (*V*) and some elements of sarcoplasmic reticulum (*SR*) are also present. Rabbit portal-anterior mesenteric vein, G/F fixation, 30 min incubation, stretch. × 28,200.

FIGURE 2 Transverse section through vascular smooth muscle cells. Smooth muscle cells have thick (thick arrow) and thin (thin arrow) myofilaments in cross section and dense bodies (*DB*), some of which are attached to depressions of the cell surface and others of which are floating free in the cytoplasm. Surface vesicles (*V*) are at regions adjacent to dense bodies. Elements of the smooth (*SR*) and rough (*R*) sarcoplasmic reticulum are present throughout the cell and are often close to mitochondria (*M*) or to the cell membrane. Rabbit small mesenteric artery, G/F fixation, 5 min incubation, no stretch. × 40,000.

truly quantitative estimate of the number of thick filaments in all preparations, due to the considerable variations encountered within the same block of tissue. For a semi-quantitative estimate, a scale of + to +++ was used to denote the numbers of thick filaments present in the whole section examined. Smooth muscle cells with very few thick filaments (+) might have been considered not to contain thick but only some electron-opaque thin filaments. However, when these scarce thick filaments were compared with those in smooth muscle cells with a moderate (++) or large (+++) number of thick myofilaments, it became clear that their structures were identical.

Drug Treatment and Contractile State

The most important combination of procedures for visualizing thick myofilaments was stretch and incubation in Krebs' bicarbonate. Thick filaments were present in tissues not stretched and not incubated (i.e. fixed directly after excision from the animal) but they were very rare. A few thick filaments were present in tissues fixed *in situ*. Thick filaments were also present in tissues which were incubated without stretch before fixation, but they were fewer than in stretched preparations (see Table I). Thick filaments were present (Table I) in theophylline- (relaxed, Fig. 10) and in norepinephrine-treated (contracted, Fig. 11) rabbit portal-anterior mesenteric veins and in portal-anterior mesenteric veins relaxed by incubation in Ca^{++}-free Krebs' bicarbonate buffer (i.e. no Ca^{++} was added) (Figs. 7, 12).

count) due to sucrose in the glutaraldehyde, however, remains to be determined, and the lower range obtained on complete micrographs of tissues fixed without sucrose is maybe the more representative value.

Longitudinally-Sectioned Myofilaments

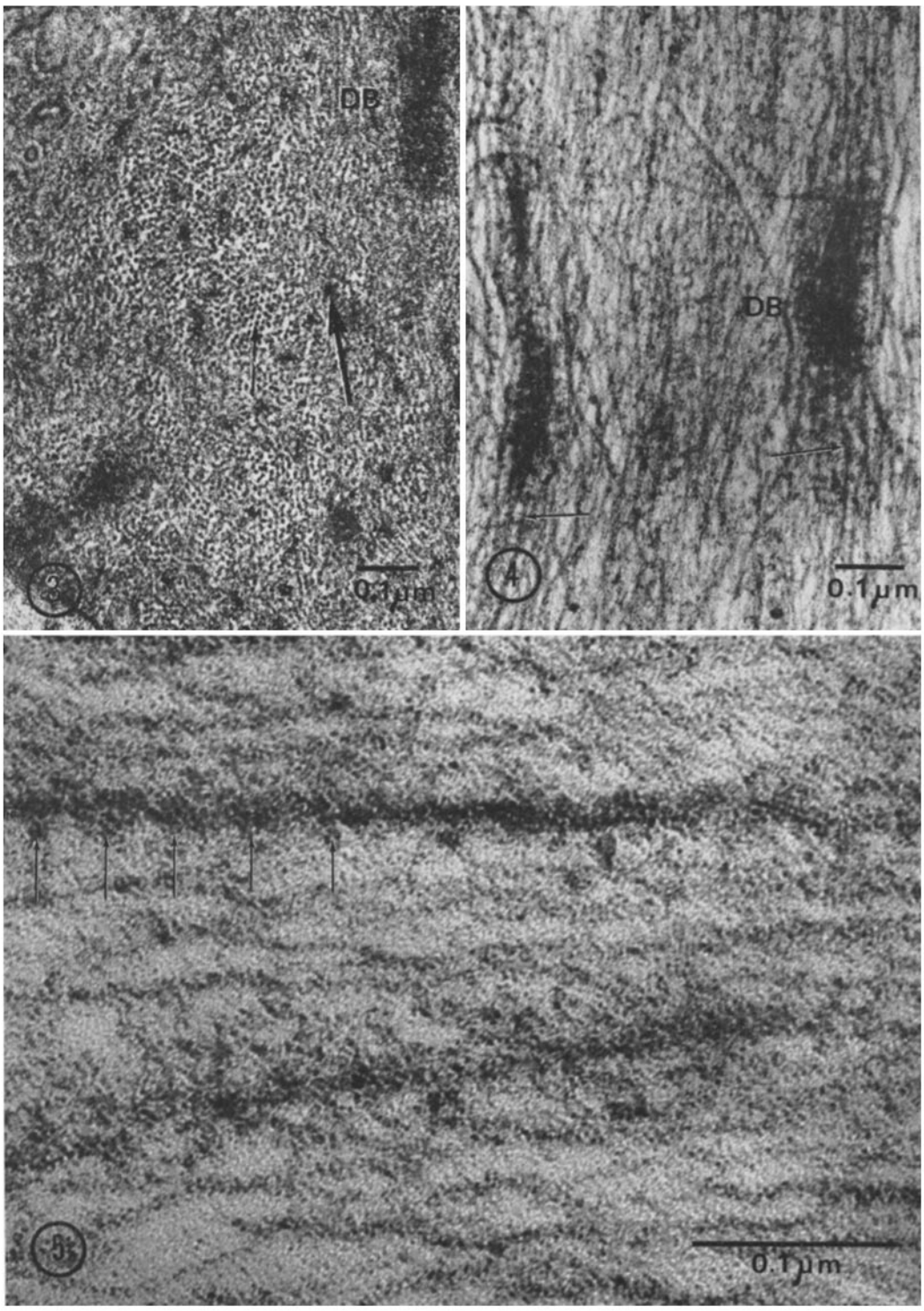
Thick and thin myofilaments were oriented along the long axis of the smooth muscle cells and were interspersed among dense bodies. Dense bodies had a maximum length of 4.5 μm in mesenteric arteries and were attached to the cell membrane in many cases (Fig. 2), but it was not possible to determine whether "free-floating" dense bodies (Figs. 2, 3, 4) were attached to the cell membrane outside the plane of section. The dense bodies have a granular substructure (12–20 nm wide), in addition to the thin filament profiles, in transverse (Fig. 3) and longitudinal sections (Fig. 4). In small mesenteric arteries, dense bodies appeared more densely stained and were in larger numbers than in other types of smooth muscles. At sites of attachment of the dense bodies, the cell membrane was often puckered and the surface vesicles were less numerous than in adjacent areas (Fig. 2, as described by Pease and Molinari, 1960). Thin filaments were attached to and ran through the dense bodies (Fig. 4). Thin filaments, although arranged predominantly along the longitudinal axis of the cell, often pursued a wavy course between comparatively straight thick filaments. They could be followed only with difficulty for large distances, because they are lightly stained and are not always in the same plane of section; hence no accurate measurements of thin filament length could be made. An unequivocal substructure of the thin filaments could not be demonstrated.

Thick myofilaments in longitudinal section

FIGURE 3 Transverse section through a smooth muscle cell containing thick (thick arrow) and thin filaments, and dense bodies (*DB*) with a granular substructure. Some thin filaments (thin arrow) are in a regular lattice. Rabbit small mesenteric artery, G/F fixation, 5 min incubation, no stretch. × 93,000.

FIGURE 4 Longitudinal section through dense bodies (DB) with a granular substructure (12–20 nm in diameter) showing thin myofilaments attached to them at the ends (thin arrows), and passing through them. Rabbit portal-anterior mesenteric vein, G/F fixation, 30 min incubation, stretch. × 102,000.

FIGURE 5 Longitudinal section showing two thick myofilaments approximately 30 nm in diameter at their widest regions, with wavy thin filaments, approximately 8 nm in diameter, between them. In one thick filament there appear to be densely-staining areas or wide lateral extensions (thin arrows). In the other filament, there is an increased electron opacity in the area adjacent to it, giving a fuzzy appearance. The wavy thin filaments occasionally come close to the lateral extensions. Rabbit portal-anterior mesenteric vein, 30 min incubation, stretch, glutaraldehyde fixation. × 306,000.


DB
0.1 μm
DB
0.1 μm
0.1 μm

ranged from 0.3 to 1.4 μm with a mean length of 0.61 μm (50 measurements) and were up to 30 nm wide at their widest regions (Fig. 5) and tapered off at either end. At high magnifications some thick filaments appeared to have densely staining areas or lateral extensions (Fig. 5). In other filaments there was an increased electron opacity in the areas adjacent to the myofilaments, producing a fuzzy appearance (Fig. 5). Thin filaments could not be seen close to the thick filaments. While thin filaments appeared to be associated with dense bodies, no clear relationship between dense bodies and thick filaments could be seen. In ultrathin sections (40 nm thick), "strips" of dense bodies with myofilaments passing through appeared very much like thick myofilaments.

Although predominantly in a longitudinal array, thick filaments were not always parallel to each other (Figs. 1, 9, 11, 12) and sometimes appeared split (Figs. 11, 14). Many instances of V-shaped junctions between thick filaments were seen (Fig. 9), but these may have been due to overlapping of thick filaments at different levels in the focal plane.

Transversely-Sectioned Myofilaments

Thick myofilaments were densely staining structures and were easily distinguished from the thin myofilaments (Figs. 2, 3, 7, 8, 10, 12, 13).

In very thin transverse sections (approximately 40 nm thick) thick filaments had irregular profiles, were electron-opaque structures, and had a granular substructure with subunits 2.5–5 nm in diameter (40 measurements) (Fig. 7).

Because of the irregularity of the structure of the myofilaments, measurements were made on micrographs at × 153,000 of both the minimum and maximum diameters of thick and thin filaments in untreated, incubated control preparations. The results, expressed as histograms in Fig. 6, show that the minimum diameters of thick filaments range from 16 to 30 nm with a mean diameter of 18 nm. The maximum diameter reaches as much as 40 nm and may indicate either lateral aggregation (see Discussion) or that some of these filaments were cut obliquely. Thin filaments have diameters ranging from 6 to 11 nm with a mean diameter of 8.4 nm. With sucrose in the fixative the mean minimum diameter of thick filaments was 16.8 nm (64 measurements); the thin filaments were more sharply defined and their mean diameter was 5.8

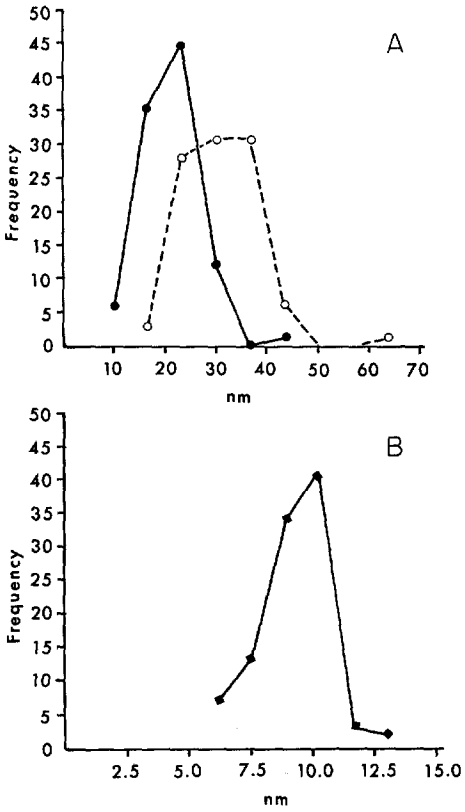


FIGURE 6 Histograms of thick and thin filament diameters in vascular smooth muscle. The measurements of thick filaments (A) are the minimum and maximum diameters of 100 filaments. The measurements of the thin filaments (B) are the maximum diameters only of 100 filaments. The mean minimum diameter of the thick filaments was 18 nm, and that of the thin filaments was 8.4 nm. Portal-anterior mesenteric vein of a rabbit stretched to 1.5 times the excised length, incubated in modified Krebs' bicarbonate solution bubbled with 95% O_2–5% CO_2, and fixed in 2% G/F. Note different scales (abscissa) in A and B.

nm (100 measurements). The closest center-to-center, thick-thin filament spacing was 13–37 nm, but at distances closer than approximately 20 nm from the center of the thick filaments the thin filaments were not easily resolved or not present (e.g. Fig. 8). Neighboring thick filaments were separated from each other by 32–100 nm at regions of the highest concentration of thick filaments. The proportion of thin to thick myofilaments ranged from 12:1 to 27:1 (e.g. Fig. 8). Both of these values are only approximations because of the difficulties in resolving the thin filaments when thick filaments

with the surrounding diffuse dense regions were near by, and because many ill defined, obliquely sectioned thin filaments were found.

DISCUSSION

Our observations revealed two distinct sets of filaments in vascular smooth muscle. Thick filaments of the dimensions found by us have not previously been documented in conventionally-fixed, mammalian vascular smooth muscle. While we can not completely identify the precise physicochemical conditions responsible for our results, it appears that stretching of the vascular strips before fixation and incubation in a physiological salt solution facilitated the demonstration of thick filaments. It is interesting to note that preincubation in some physiological medium was also part of the experimental protocol in some studies that attributed visualization of thick filaments to experimental variables other than the incubation itself (Cooke et al., 1970; Kelly and Rice, 1969), although thick filaments have also been demonstrated in nonincubated mammalian smooth muscle (Bennett and Cobb, 1969; Nonomura, 1968; Yamauchi and Burnstock, 1969).

Contrary to the reports of others, neither externally added adenosine triphosphate (ATP) (Kelly and Rice, 1969) nor electrophoretic extraction of ions from smooth muscle (Cooke et al., 1970) was necessary for the demonstration of thick myofilaments. Furthermore, within the range of uncertainty of any electron microscope survey, the number of thick filaments and the ease of demonstrating them did not vary whether the muscles were contracted with norepinephrine or relaxed by theophylline or in Ca^{++}-free media. The latter observation is not necessarily inconsistent with the divalent cation requirements for *isolating* thick filaments (Kaminer, 1969; Shoenberg, 1969), as it is unlikely that the intracellular concentration of free Ca^{++} in *relaxed* vascular smooth muscle is materially affected by the omission of Ca^{++} from the external medium. The unequivocal demonstration of thick filaments in relaxed vascular smooth muscle seems to preclude their formation at the time of excitation–contraction coupling just before the onset of contraction, and suggests that they are stable structures in relaxed and in contracted mammalian vascular smooth muscle.

The question arises whether the thick filaments demonstrated in our study are true myofilaments present in vivo or whether they are some preparatory artifact due to the coalescence of thin, actin filaments. It should be stated at the outset that the present experiments cannot definitely identify the thick filaments as being constituted of myosin. At most, we can suggest that, in our preparations showing the largest number of thick myofilaments, we can rule out the objection to earlier studies that the number of thick filaments present are not sufficient to account quantitatively for the biochemically demonstrable myosin (Needham and Shoenberg, 1964). There also appeared to be some regularity of the lateral spacing of the thick filaments, which would militate against their being formed as an artifact by random coalescence of thin myofilaments. The diameter of the thick myofilaments is not a multiple of that of thin myofilaments, contrary to the objections of Panner and Honig (1970) regarding thick myofilaments demonstrated by others. Under high magnification the particles constituting the thick filaments, when measured in cross section, are smaller (2.5–5 nm) than actin subunits (5.5 nm) or the thin filament diameters (8.4 nm) observed in our material and are similar to those shown in thick filaments of striated muscle (Baccetti, 1967; Pepe, 1971). Furthermore, unlike thin filaments, the thick myofilaments demonstrated do not connect with any degree of frequency to the dense bodies. For these reasons, we believe that the thick myofilaments illustrated in this study do not represent coalescence of thin (actin) myofilaments. We do not feel that sectioned material is suitable for the demonstration of the globular subunits of actin filaments.

The details of the substructure of thick myofilaments *in situ* remain to be determined. It would appear likely that the myosin filament in mammalian smooth muscle is a labile structure that is readily disturbed during fixation. It should be noted, in this regard, that even the very readily identifiable thick myofilaments of the retractor penis muscle of *Helix pomatia* decrease in number and increase in cross-sectional area when subjected to incubation and repeated passive stretch-release cycles (Foh, 1969). It is very likely that the incubation procedures employed by us also lead to some disturbance of the myosin filament substructure and that the filament profiles (e.g. Fig. 7) represent a spectrum of almost normal and partially dispersed thick myofilaments. The apparent splitting of thick myofilaments (Figs. 11, 14) may be due to disaggregation, but could also

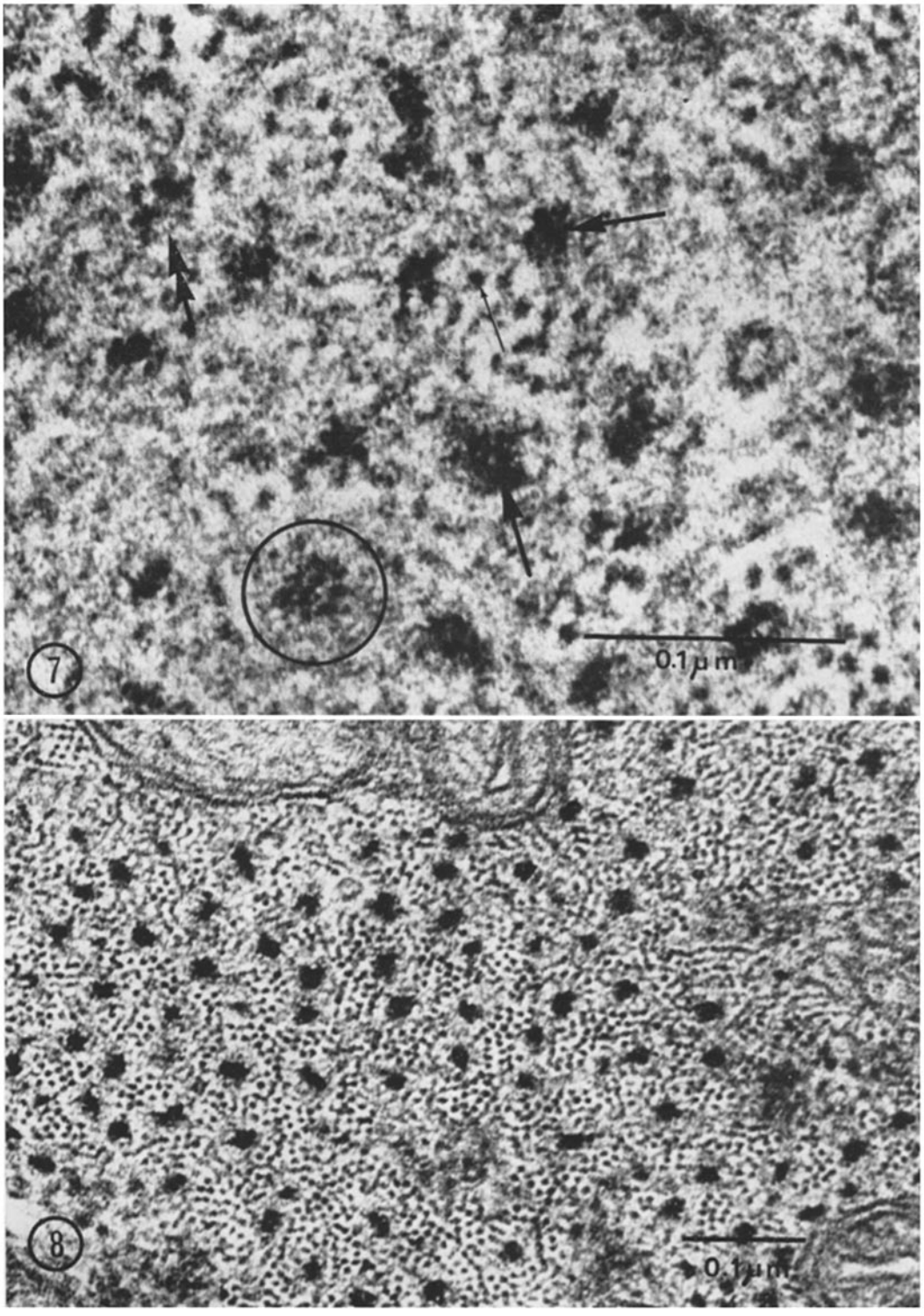
reflect the hollow cores seen in the thick myofilaments of freeze-substituted, vascular smooth muscle (Pease, 1968) as well as in a number of conventionally fixed, striated muscles (for review see Pepe, 1971). Variations in density in longitudinally sectioned material may correspond to regions of cross bridges.

The diameter and the length of the thick filaments observed in this study were not significantly different from the respective parameters of synthetic (Kaminer, 1969) or isolated (Shoenberg, 1969) myosin filaments obtained from chicken gizzard muscle, under optimal conditions of producing synthetic or isolated filaments. Direct comparison of the latter studies with our results, however, is not entirely feasible, because, as pointed out elsewhere (Somlyo and Somlyo, 1968), the myosins isolated from different vertebrate smooth muscles may differ biochemically. Furthermore, it is likely that, since "perfect" longitudinal sections including both ends of filaments are difficult to obtain, our values underestimate true filament length. It is also entirely possible that myosin filaments in different smooth muscles among various species, or even within different organs of the same species, do not have the same ultrastructure.

While this study was being concluded, Lowy and Small (1970) had reported that myosin is organized into ribbon-like structures in the guinea pig taenia coli. Their preparations were incubated, at 0°C, for even longer periods than ours (4 hr) and were stretched by an amount (10 g) that would seem to exceed the tension per cross-sectional area applied in our study. Our experiments with less stretch produced circular profiles, in the presence of regular thin filament arrays (Figs. 3, 8), as have also been demonstrated in taenia coli by Rice and his coworkers (1970). The preparatory techniques we found suitable for the demonstration of thick filaments, incubation in oxygenated Krebs' solution at 37°C for 30 min under stretch of approximately 1.5 times the excised length, are identical to those used successfully for several years in our laboratory in pharmacological and physiological studies of vascular smooth muscle. Vascular smooth muscle in vitro under these conditions maintains a stable resting membrane potential, generates action potentials, and exhibits normal contractile and electrical responses to drugs (for references see Somlyo and Somlyo, 1968; 1970). On the other hand, it is very unlikely that mammalian smooth muscle would be able to contract under the extreme conditions of stretch and low temperatures employed in the study of Lowy and Small (1970). Although we cannot rule out the possibility that the latter authors' preparatory techniques produce better preservation of in vivo ultrastructure, nevertheless it seems more likely that fixation in a physiologically contractile state would more faithfully reflect the ultrastructure of functional contractile elements. The ribbon-like structures demonstrated by Lowy and Small (1970) probably represent lateral aggregation of thick myofilaments, perhaps due to the extreme stretch which removes the interdigitating thin filaments from the array of thick myofilaments and, in a constant volume lattice, with decreasing interfilament distance, promotes lateral aggregation. In the anterior byssus retractor muscle of *Mytilus* incubated in a physiological salt solution under ten-

FIGURE 7 High magnification view of transversely-sectioned thick filaments with thin filaments (thin arrow) in between them. The thick filaments have a granular substructure and subunits 2.5–5 nm in diameter. Several densely-staining thick filaments are present (thick arrows) but some thick filaments appear to be partly dissociated (double-headed thick arrow). One profile (circled) suggests the presence of the two central subfilaments surrounded by a circle of additional subfilaments, as described in striated muscle (Baccetti, 1965; and Pepe, 1971). The closest center-to-center spacing of thick filaments is approximately 43 nm. Rabbit portal-anterior mesenteric vein, 30 min incubation in Ca^{++}-free Krebs', stretch, G/F fixation. × 392,700.

FIGURE 8 High magnification view of transversely-sectioned thick and thin filaments. The closest center-to-center thick and thin filament spacing is approximately 15 nm, but usually there is an amorphous electron-opaque area around each thick filament where no thin filaments are present. The center-to-center spacing between thin filaments is approximately 8–11 nm. At the regions of high filament density, the ratio of thin to thick filaments is 12:1. Rabbit portal-anterior mesenteric vein, 30 min incubation, stretch, glutaraldehyde-sucrose. × 186,000.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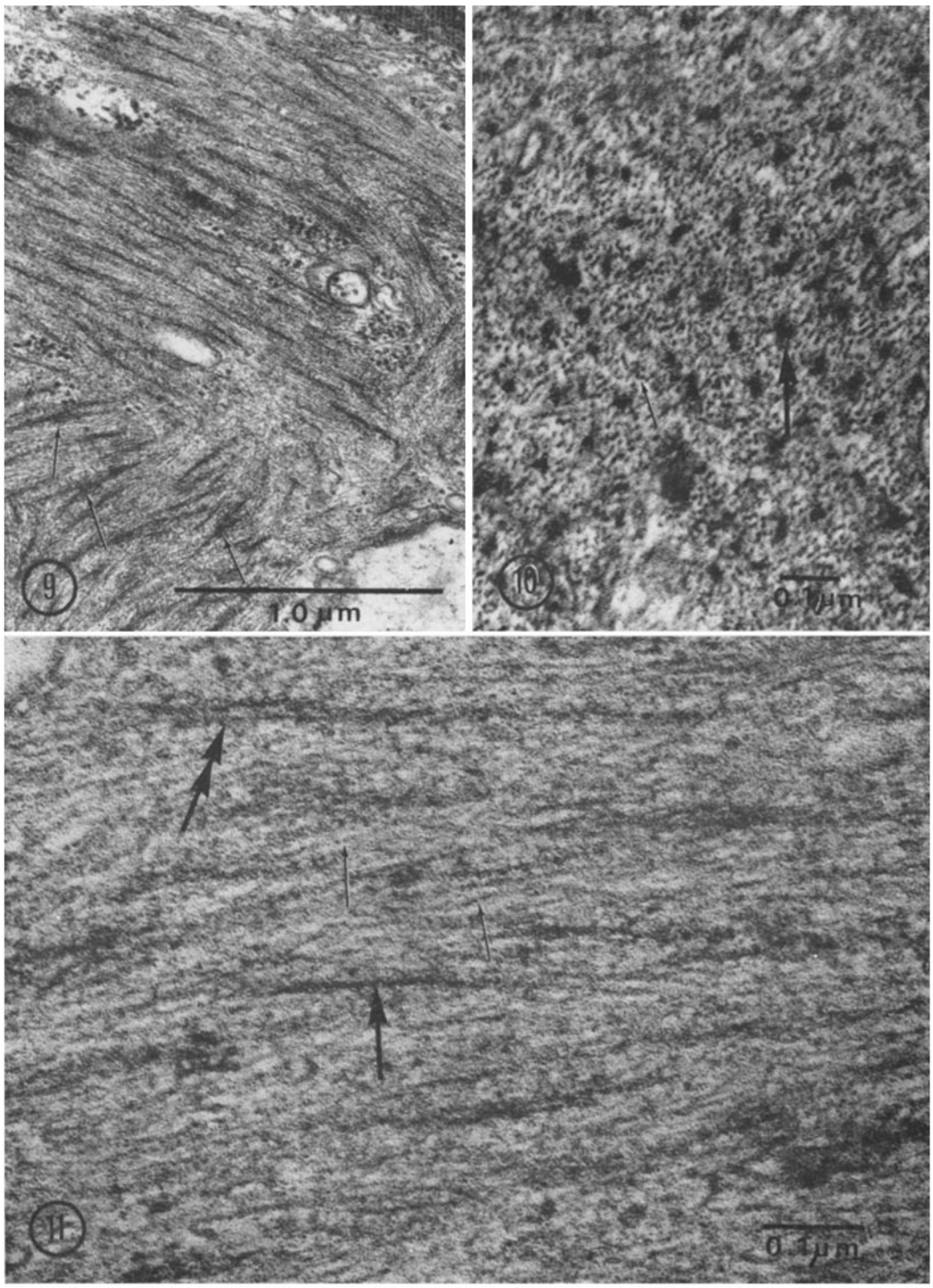

FIGURE 9 Nonparallel, longitudinally-sectioned thick filaments fusing with each other to form V-junctions at several regions (thin arrows). Rabbit portal-anterior mesenteric vein, 30 min incubation, stretch, G/F fixation. × 40,000.

FIGURE 10 Transversely-sectioned thick (thick arrow) and thin (thin arrow) filaments in normal numbers, after treatment with a relaxing agent, theophylline. Rabbit portal-anterior mesenteric vein, 30 min incubation including final 5 min in 2.5 mM theophylline, stretch, G/F fixation. × 90,000.

FIGURE 11 Longitudinal section of thick filaments showing "split" thick myofilaments (double-headed thick arrow) in an ultrathin section. Lightly-staining thin filaments (thin arrows) are present between the thick filaments (thick arrow). Rabbit portal-anterior mesenteric vein, 30 min incubation including final 5 min in 10 μg/ml norepinephrine, G/F fixation. × 153,000.

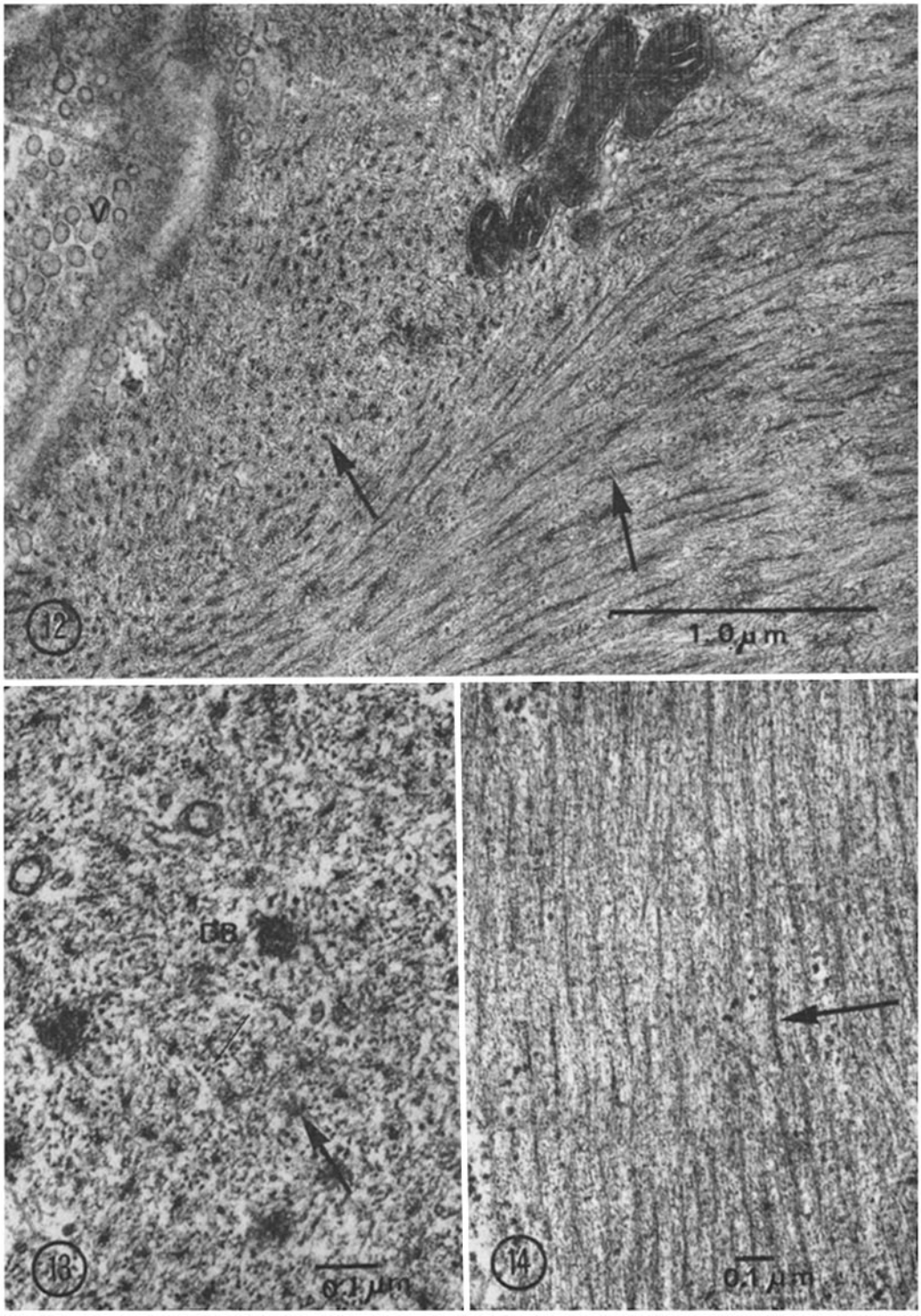

FIGURE 12 The variable orientation of thick and thin myofilaments in the same smooth muscle cell is indicated by the transversely and longitudinally-sectioned thick (thick arrows) and thin filaments in normal numbers. Rabbit portal-anterior mesenteric vein, 30 min incubation, Ca^{++}-free medium, stretch, G/F fixation. × 40,000.

FIGURE 13 Transversely-sectioned thick (thick arrow) and thin (thin arrow) filaments in the vas deferens. Dense bodies (*DB*) are also present. Rabbit vas deferens, 30 min incubation, stretch, G/F fixation. × 93,000.

FIGURE 14 Longitudinally-sectioned thick (thick arrow) and thin filaments in the taenia coli. Rabbit taenia coli, 30 min incubation, stretch, G/F fixation. × 60,000.

sion, lateral aggregation of thick myofilaments has been demonstrated (Heumann and Zebe, 1968). Close aggregation of thick filaments was noted by Rosenbluth (1967) in obliquely striated muscle of *Ascaris*. Further studies will be necessary to determine with complete certainty whether the thick filament form or the ribbon-like structures suggested by Lowy and Small (1970) represent the correct form of organized myosin in mammalian smooth muscle. The X-ray diffraction studies of Lowy et al., (1970), however, lend strong support to the assumption that the thick myofilaments demonstrated in these and in previous studies do indeed represent the myosin organized in mammalian smooth muscle.

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Note Added in Proof: Optical transforms of electron micrographs of rabbit portal-anterior-mesenteric vein in transverse section reveal thick filaments arranged in a quasi-rectangular array at a popular spacing of approximately 700 A (Rice, R. V., McManus, G. M., Devine, C. E., and Somlyo, A. P. Nature, (*London*), in press, and Biophysical Society Abstracts, 110 a: 1971). This normal arrangement of thick filaments is disturbed by excessive stretch and incubation of the smooth muscle in hypertonic solutions, leading to aggregation of thick filaments into ribbon-like structures (Somlyo, A. P., Somlyo, A. V., Devine, C. E., and Rice, R. V. Nature, (*London*), in press, and Biophysical Society Abstracts, 110 a: 1971).

We also observed thick filaments, in numbers comparable to those found in the other smooth muscles reported here, in a guinea pig uterus stretched in Krebs solution before fixation in glutaraldehyde buffered with cacodylate and containing 6% sucrose, in an embedding completed after acceptance of this manuscript. Thick filaments have also been demonstrated in rabbit (Rice et al., Biophysical Society Abstracts, 110 a: 1971) and *human* (Somlyo et al., Nature, (*London*), in press) uterine smooth muscle. The results suggest that demonstration of the normally present thick filaments in mammalian smooth muscle is primarily a problem of adequate preservation for electron microscopy.

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