Dense Bodies and Actin Polarity in Vertebrate Smooth Muscle

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ABSTRACT   The arrangement of cytoplasmic dense bodies in vertebrate smooth muscle and their relationship to the thin filaments was studied in cells from rabbit vas deferens and portal vein which were made hyperpermeable (skinned) with saponin and incubated with myosin subfragment 1 (S-1).

The dense bodies were obliquely oriented, elongated structures sometimes appearing as chains up to 1.5 μm in length; they were often continuous across the cell for 200 to 300 nm and were interconnected by an oblique network of 10-nm filaments.

The arrowheads, formed by S-1 decoration of actins, which inserted into both the sides and ends of dense bodies, always pointed away from the dense body, similar to the polarity of the thin filaments at the Z-bands of skeletal muscle.

These results show that the cytoplasmic dense bodies function as anchoring sites for the thin filaments and indicate that the thin filaments, thick filaments, and dense bodies constitute a contractile unit.

The role of the cytoplasmic dense bodies in vertebrate smooth muscle (33), their relationship to actin filaments and the manner in which the thin (actin) and thick (myosin) filaments are organized into contractile units (1, 8, 48) has been the subject of some debate. There is considerable support for the view that the surface dense bodies, or plaques, act as anchoring sites for the thin filaments (5, 16, 18, 33, 40, 48, 51). By parallax measurements (1), in ultrathin sections (1, 53), and in isolated dense bodies (35, 39), it has also been demonstrated that thin filaments insert into the cytoplasmic dense bodies. On the basis of this evidence, and in view of the established role of the cytoplasmic dense bodies as anchoring sites for thin filaments in invertebrate smooth muscle (20, 53), it has been proposed (1, 5, 44) that the cytoplasmic dense bodies have a similar function to the surface dense bodies i.e. they are functionally analogous to the Z-bands of striated muscle. Others (8, 46, 47) however, have proposed that the cytoplasmic dense bodies function as attachment points or nodes for a cytoskeletal network of 10-nm filaments, and that this constitutes a system distinct and separate from the contractile apparatus of thin and thick filaments. This view is based on the observed association of 10-nm filaments with both cytoplasmic and surface dense bodies (1, 8, 9, 10) and the persistence of a network of 10-nm filaments throughout cells from which actomyosin has been extracted (46, 47).

In the experiments to be described in this paper, we demonstrate that cytoplasmic dense bodies act as anchoring points for the thin filaments, as previously proposed (1, 39, 51), and that the contractile units, consisting of thick and thin filaments and dense bodies, are interconnected by the 10-nm filament network.

Our investigation of the role of the cytoplasmic dense bodies depends on the identification of the polarity of the thin filaments. Polarity is determined by decoration of actin in the smooth muscle cell with myosin subfragment 1 (S-1) (32). If the dense bodies act as anchoring sites for the thin filaments, the thin filaments that insert into the dense bodies (determined by viewing the dense bodies in stereo) should have opposite polarity at each end, i.e. the arrowheads formed upon S-1 decoration will always point away from the dense body. This approach was originally used by H. E. Huxley (23) who used heavy meromyosin to demonstrate the polarity of thin filaments with respect to the Z-band in isolated I-bands of skeletal muscle. It has also been used to determine actin polarity in isolated dense body-actin preparations from molluscan smooth muscle (53). The technique has been adapted for the observation of arrowhead complexes in glycerinated (25) or Triton-extracted cells (54, 55). The smooth muscle cell membrane can be rendered permeable to macromolecules by chemical skinning with saponin (12, 37) thus allowing penetration of the

myosin S-1. Intracellular structure is maintained and maximal tension development is similar to that in unskinned preparations (13, 25, 34, 43, 49).

This work has been presented in preliminary form (6, 52).

MATERIALS AND METHODS

Male New Zealand rabbits (2–3 kg) were killed by a blow on the head.

Longitudinal strips (1-mm wide) of portal-anterior mesenteric vein (PAMV) and vas deferens were obtained, cleaned of adventitia and stretched to approximately 1.75 times slack length. The strips were incubated in modified Krebs buffer (NaCl 125.1 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 18.7 mM, $CaCl_2$ 1.2 mM, dextrose 5.6 mM) and bubbled with 95% O_2, 5% CO_2 for 90 min at 37°C.

S-1 Decoration of Thin Filaments

Incubations with S-1 were carried out in either a low ionic strength solution (solution A) or a solution of ~90% normal ionic strength (solution B). Neither of these solutions contained ATP, thus preventing dissociation of the actin-S-1 complexes. Strips of PAMV and vas deferens were processed by one of the following procedures: (a) The strips were rinsed in a low ionic strength KCl solution containing no divalent cations, to slightly swell the filament lattice, (KCl 75 mM, EGTA 10 mM, PIPES 10 mM, dextrose, 5.6 mM, pH 7.1) (Solution A). They were then treated with saponin (200 μg/ml) in solution A for 60 min at 25°C and rinsed in solution A. The strips were transferred to myosin S-1 (2 mg/ml) in solution A for 5 h at 0°C and then rinsed in solution A for 20–30 min. (b) Nonswollen smooth muscle cells were prepared by rinsing strips of muscle in a KCl solution of higher ionic strength (KCl 80 mM, PIPES 20 mM, dextrose 5.6 mM, MgCl 1.5 mM, $CaCl_2$, 1.2 mM, EGTA 10 mM, pH 7.2) (Solution B). They were then fixed in paraformaldehyde (1%) in solution B for 30 min at 25°C. The strips were treated with saponin (in solution B) rinsed in solution B then incubated in S-1 in solution B, as before, with a final rinse in solution B for 20–30 min. (c) Control (nondecorated) cells were prepared by rinsing strips in solution C. (K methanesulfonate 80.3 mM, PIPES 20 mM, dextrose 5.6 mM, $MgCl_2$ 1.3 mM. EGTA 10 mM, and protease inhibitors—aprotinin 5 μg/ml, benzamidine 5 mM, PMSF (α-toluene sulfonyl fluoride) 0.1 mM, leupeptin 5 μg/ml, pH 7.0). Strips were then incubated in saponin (50 μg/ml) for 45 min at 25°C in solution C and incubated in solution C at 0°C for 5 h.

Electron Microscopy

Strips of PAMV and vas deferens that had been incubated in solutions A, B, and C were fixed in 2% glutaraldehyde + 2% tannic acid for 90 min. The strips were postfixed in 2% OsO_4, stained en bloc in aqueous uranyl acetate, dehydrated in ethanol and embedded in Spurr resin. All fixation and embedding steps were carried out at 25°C. Thin sections (60–70 nm) were cut and stained with Reynolds Pb citrate. Sections were examined on a rotating goniometer stage in a Philips 400T electron microscope at an accelerating voltage of 80 kv. Pairs of stereo photomicrographs were taken at tilt angles of ±12° or ±18°.

Tracings from Serial Sections

Surface and cytoplasmic dbs only were traced on clear plastic sheets from electron micrographs of consecutive longitudinal 80 nm sections of non saponin-treated PAMV at a magnification of 22,500 times (serial thin sections cut by Dr. F. T. Ashton, University of Pennsylvania). The sheets were then superimposed exactly and photographed. The composite photograph was used as one of two in a stereo pair. The second photograph of the pair consisted of the same tracings with each tracing shifted horizontally by 0.5 mm with respect to the tracing below it. The pair of composite photographs was then viewed in stereo and have been reduced for publication (Fig. 3). The percentage of the cell cross-sectional area occupied by cytoplasmic dense bodies was determined by using a microprocessor-linked digital planimeter designed by L. D. Peachey (41).

RESULTS

In transverse sections of smooth muscle cells, the cytoplasmic dense bodies are typically densely staining structures of roughly circular or oval shape scattered throughout the cytoplasm and surrounded by transverse profiles of 10-nm filaments (1, 8, 46, 56, for review, see reference 50). The circular profiles represent slices through elongated structures obliquely oriented to the long axis of the cell, rather than spheres, as illustrated in the

transversely sectioned cell shown in Fig. 1, where the filaments and two dense bodies on the left are running obliquely. Occasionally, in longitudinal sections, chains of dense bodies connected by 10-nm filaments appeared with actin filaments inserting at both ends as well as along the entire length of the elongated dense body. This is clearly demonstrated in the saponin-skinned cells in Fig. 2.

The configuration of the dense bodies has been examined in more detail by the reconstruction of longitudinally oriented serial sections. Most of the dense bodies were continuous through three to four 80-nm sections (arrows), but were displaced relative to each other. This obliquely oriented, elongated form of the dense bodies as well as their number and distribution throughout the cytoplasm can be seen in the five cells shown in the stereo pair of the reconstructed serial sections in Fig. 3. This low magnification reconstruction is composed from tracings made from 13 80-nm thick serial longitudinal sections (~1.0 μm of cell thickness). Only cytoplasmic and surface dense bodies have been traced. In some regions, dense bodies appeared to be strung out in chains up to 1.5 μm in length, some
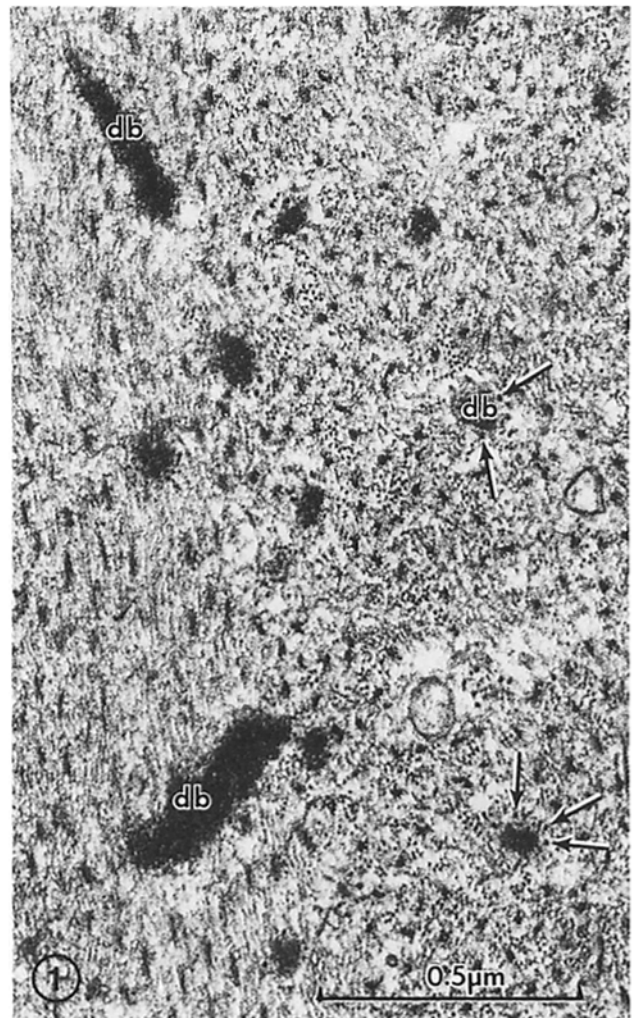

FIGURE 1 Transverse section of a vas deferens smooth muscle cell. Cytoplasmic dense bodies (db) are usually round or oval in shape and surrounded by 10-nm filaments (arrows). In the center of the micrograph there are two dense bodies which are obliquely sectioned and have a more elongated shape. Obliquely sectioned thin filaments appear to originate from these latter dense bodies. × 70,000.

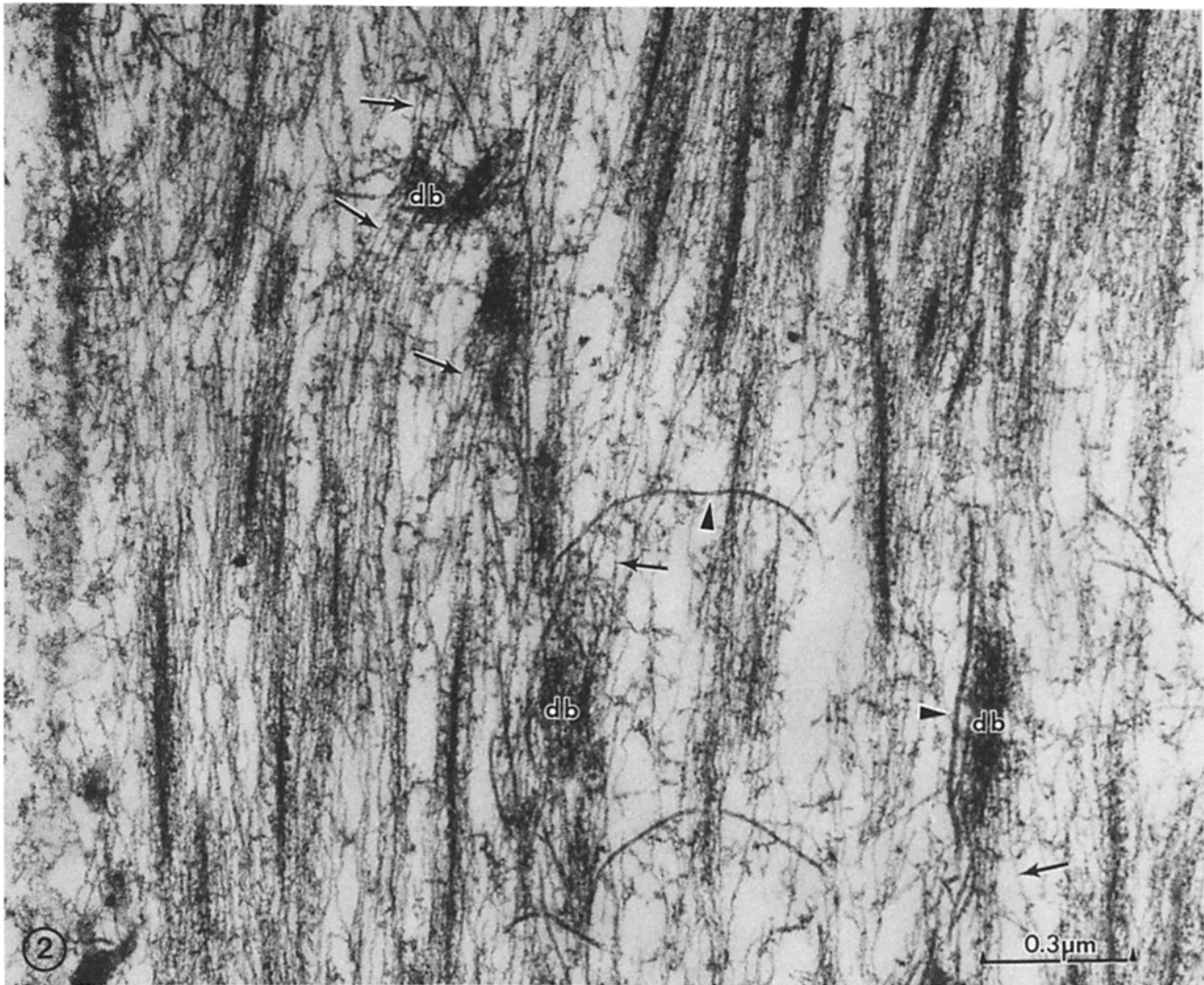FIGURE 2 Longitudinal section of a portion of a PAMV smooth muscle cell. The cells were saponin-treated (50 μg/ml) in relaxing solution (10^{-7} M Ca^{2+}, 4 mM MgATP, pH 7.1) for 30 min and then fixed with 2% glutaraldehyde for 90 min in the presence of 0.1% tannic acid. Thin filaments (arrows) insert into both sides of the dense bodies (*db*); 10-nm filaments (arrowheads) sometimes interconnect a series of dense bodies to form a chain. × 70,000.

of which are obliquely oriented, and terminate at the cell membrane. The central region of the cell in the center of Fig. 3 has few dense bodies: this space was occupied by the nucleus. The surface dense bodies (arrowheads) were frequently superimposed from one section to the next, but their pattern of distribution was not apparent, largely because the information was obscured in the superposition of the traces. The volume of organelle-free region of the cell occupied by dense bodies was determined by planimetry from these traces to be 3.6 to 4.7% (from the three central cells averaged over four successive sections).

In muscles exposed to saponin, breaks were frequently observed in the cell membranes as noted by Ohtsuki et al. (37). The excellent preservation and staining of saponin-treated cells is likely to be due to the easy penetration of the fixative and tannic acid (1, 3). The clarity of the surrounding cytoplasm (especially Figs. 2, 6, and 12) suggests that soluble cytoplasmic proteins are washed out during and after saponin treatment, revealing the filaments more clearly. Occasionally, in cells such as shown in Figs. 2 and 12, the bundles of filaments have separated revealing the dense bodies with actin filaments

emerging from both sides of single dense bodies (Fig. 12) as well as from chains (Fig. 2). Transverse sections of similar cells are shown in Fig. 6, in which a normal myosin interfilament spacing, actin/myosin ratio, and the typical rosettes of actins surrounding myosins are shown. Fig. 4 shows a transverse section of saponin-skinned smooth muscle cells of portal vein which was kept at 0°C in solution C (in the absence of S-1) for the same period as the S-1 treated tissues (5 h). It can be seen that after saponin-skinning and then several hours of incubation, the thin filament number, orientation, and distribution appear normal. A similar number of cytoplasmic dense bodies surrounded by intermediate filaments is found per cell cross-sectional area as in cells from nonskinned smooth muscle. Regular arrays of thick filaments are also present.

Smooth muscle cells that were treated with S-1 for 5 h after saponin-skinning also contained a well-ordered thin-filament lattice as shown in cells from tissue incubated in Solution B (Fig. 5). Thick filaments were also usually present in these cells. However, the myosin filaments were often lost during the 5-h incubations with S-1 at pH 7.2 in the low ionic strength solution (Solution A).
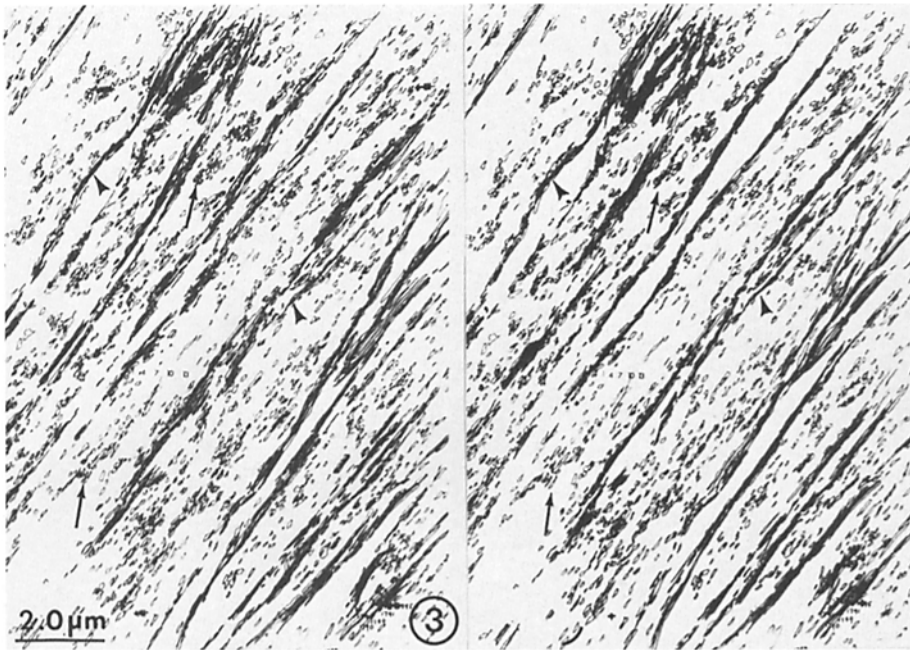
FIGURE 3 Three dimensional projection of cytoplasmic and surface dense bodies obtained from serial longitudinal sections of PAMV, which was not saponin-treated. The surface and cytoplasmic dense bodies were traced from 13 consecutive 80-nm serial sections. Arrows point to some of the dense bodies that can be followed through several serial sections. The apparent gaps between superimposed dense bodies is, in most cases, a result of the method used in obtaining the three-dimensional image (see Materials and Methods section). If dense body outlines in adjacent sections overlap, the dense body is continuous. Chains of dense bodies often converge on surface dense bodies. The latter are indicated by arrowheads. × 65,000.
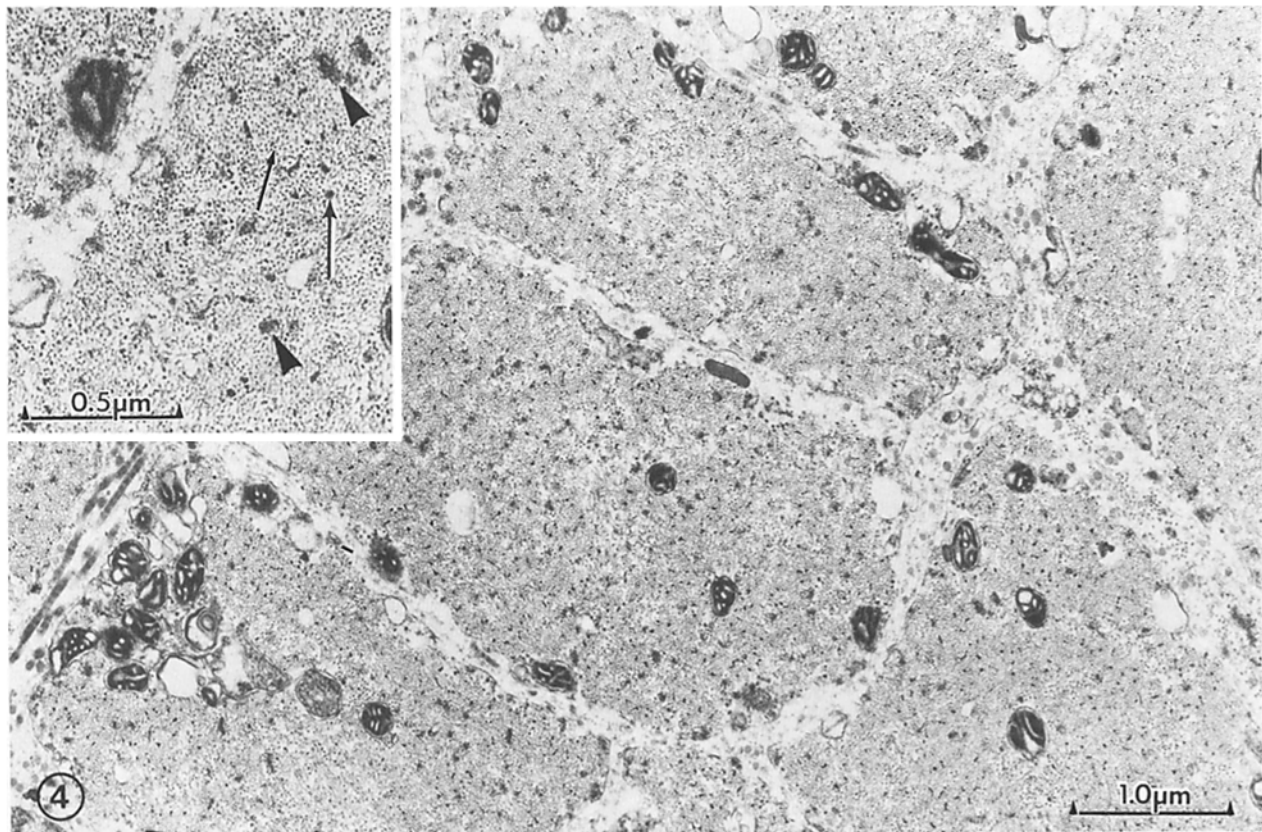

FIGURE 4 Transverse section of rabbit PAMV skinned 45 min in saponin (50 µg/ml) in solution C followed by 5 h at 0°C in solution C. Regular arrays of thin (small arrow) and thick filaments (large arrows) are present. Dense bodies (arrowhead) are seen surrounded by intermediate filaments. × 21,000. *Inset,* × 42,000.

Upon incubation with myosin S-1, the decorated thin filaments were easily observed in longitudinal sections at low magnification (Fig. 7). The number of decorated cells was highly variable. At higher magnifications the typical polarized arrowhead structures on the thin filaments entering the cytoplasmic dense bodies were resolved (Figs. 8 and 9). The axial periodicity of the arrowheads measured along actin filaments

in four cells was 36 nm and the angle of the arrowheads was ~30°. The arrowheads were more easily seen in the swollen (Figs. 8 and 9) than in the nonswollen (Fig. 10) cells due to greater filament separation in the former. Actin polarity was determined in both swollen and nonswollen preparations. Examination of stereo pairs revealed that the decorated actin filaments inserted into the dense bodies, rather than coursing
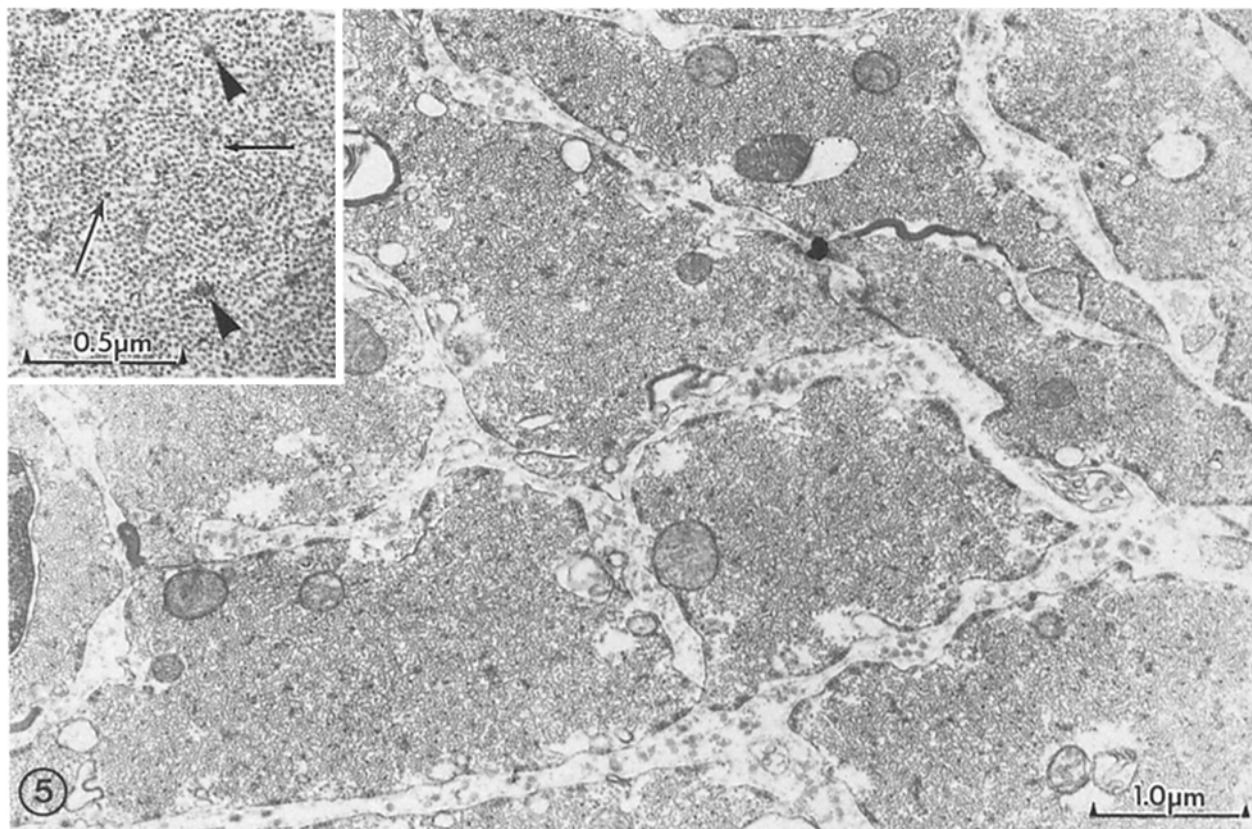
FIGURE 5   Transverse section of rabbit vas deferens, saponin-skinned 45 min (200 μg/ml) in solution B and then incubated with S-1 for 5 h at 0°C in solution B. S-1-decorated thin filaments (small arrow), thick filaments (large arrow), and dense bodies (arrowhead) are seen with surrounding intermediate filaments. × 21,000. *Inset,* × 42,000.

above or below them. The number of filaments for which the direction of the arrowheads could be resolved was greatly enhanced by stereoscopic viewing. In thin sections (<70 nm) of vas deferens and PAMV the average number of decorated actin filaments/dense body,[1] emerging from one side was 4.2 ± 0.3 SEM (n = 72 dense bodies). Of the 72 dense bodies examined, 66 had decorated actin filaments emerging from both ends. In the other six dense bodies, five of which were found in nonswollen cells, there was too much overlap of the decorated filaments to determine whether or not they emerged from the dense body. The percentage of actin filaments emerging from dense bodies having arrowheads which pointed away from the dense body, was 94% (n = 328). (In the other 6%, polarity could not be determined due to filament superposition.) The arrowheads on the actin filaments emerging from the sides of the very elongated dense bodies were also directed away as illustrated in Fig. 10. The results of examination of many other images which were not viewed in stereo or tabulated were consistent with the above data. We did not find any evidence for dense bodies that were not associated with actin filaments.

In some views, an amorphous material appeared to surround the base of the thin filaments where they entered the dense bodies. Arrowheads pointed away from the surface membrane on the actins which originated at the surface dense bodies.

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[1] The value of 4.2 does not represent the total number of actins/dense body but only those within the 60–70 nm longitudinal slice of dense body in the section and only those for which the arrowheads were clearly resolved.

The 10-nm filaments appeared to be connected by amorphous bridging material to the cytoplasmic dense bodies when viewed in stereo, in very thin (40 nm) longitidunal sections of nontreated smooth muscle cells (Fig. 11). This can be observed in the labeled dense body on the far right of Fig. 2 and is also evident in transverse sections of dense bodies (plate XIV of reference 1).

The 10-nm filaments were not as tightly associated with the dense bodies in the swollen as in the nonswollen cells although in transverse sections they still surrounded the majority of dense bodies as in unskinned cells.

DISCUSSION

The entry of actin filaments into cytoplasmic dense bodies was particularly clearly revealed in the saponin-skinned smooth muscles used in the present study, and is in agreement with previous observations made on stereo views of longitudinal sections and on ultrathin transverse sections of dense bodies (1). The present results show, in addition, that virtually all of the cytoplasmic dense bodies are associated with actin filaments. Furthermore, decoration with myosin S-1 demonstrated that the thin filaments associated with all of these dense bodies are polarized, with opposite polarities on either side of a given dense body. We conclude, as suggested previously (1, 39, 44, 51), that the cytoplasmic dense bodies are functionally equivalent to the Z-bands of striated muscle and that other, contrary views (8, 47) are incorrect.

The serial reconstructions of the dense bodies from longitudinal sections show the cytoplasmic dense bodies to be elongated, obliquely oriented structures. This shape gives rise to the
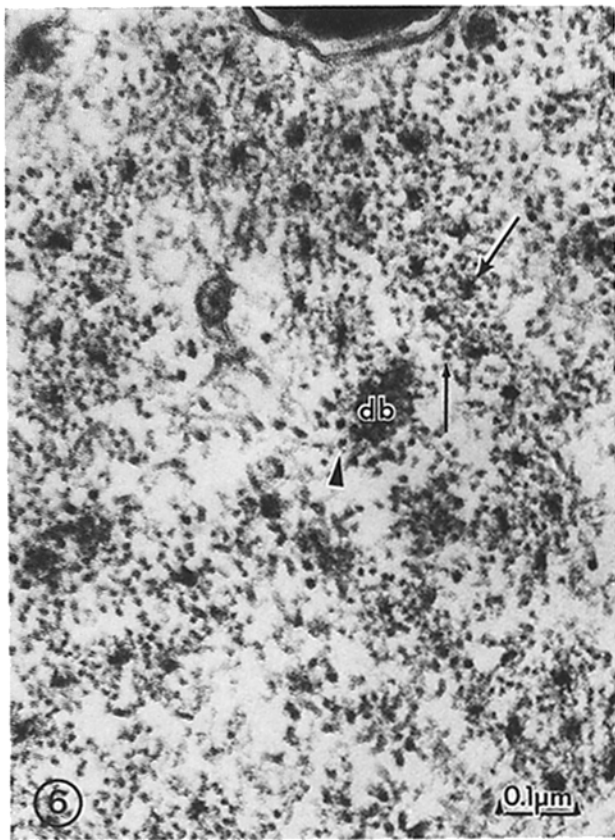
typical round or oval outline in transverse sections. In single longitudinal sections parallel to the plane of the long axis of a dense body at its greatest width, the elongated form will be observed (e.g. dense body in Fig. 10), whereas in longitudinal sections perpendicular to the plane of the long axis of the dense body, the short form will be seen (labeled dense body on the right, Fig. 12). Sections through other planes will give rise to intermediate forms. The dense bodies followed in the serial reconstructions were commonly continuous through a depth of approximately 200–300 nm and were up to 1.5 µm in their longest dimensions.

The volume reconstructed is not sufficient to show an ordering of the dense bodies and if it does exist it may require the reconstruction of entire cells. The ordering ultimately must accommodate 2.2 µm longitudinally oriented myosin filaments between two oblique dense bodies and their related actin filaments. Fay has recently described the distribution of dense bodies in toad stomach smooth muscle cells stained with α-actinin antibodies (14, 15).

The granular periodic material linking the actin filaments within the dense bodies (Fig. 11) and/or the amorphous material seen at the base of some of the actin filaments as well as

FIGURE 6 Transverse section of rabbit PAMV, saponin-skinned in relaxing solution as in Figs. 2 and 12 showing rosettes of actin filaments (small arrow), which are well-aligned and longitudinally oriented to the cell axis, surrounding myosin filaments (large arrow). The dense body (db) is surrounded by 10-nm filaments (arrowhead). × 108,000.
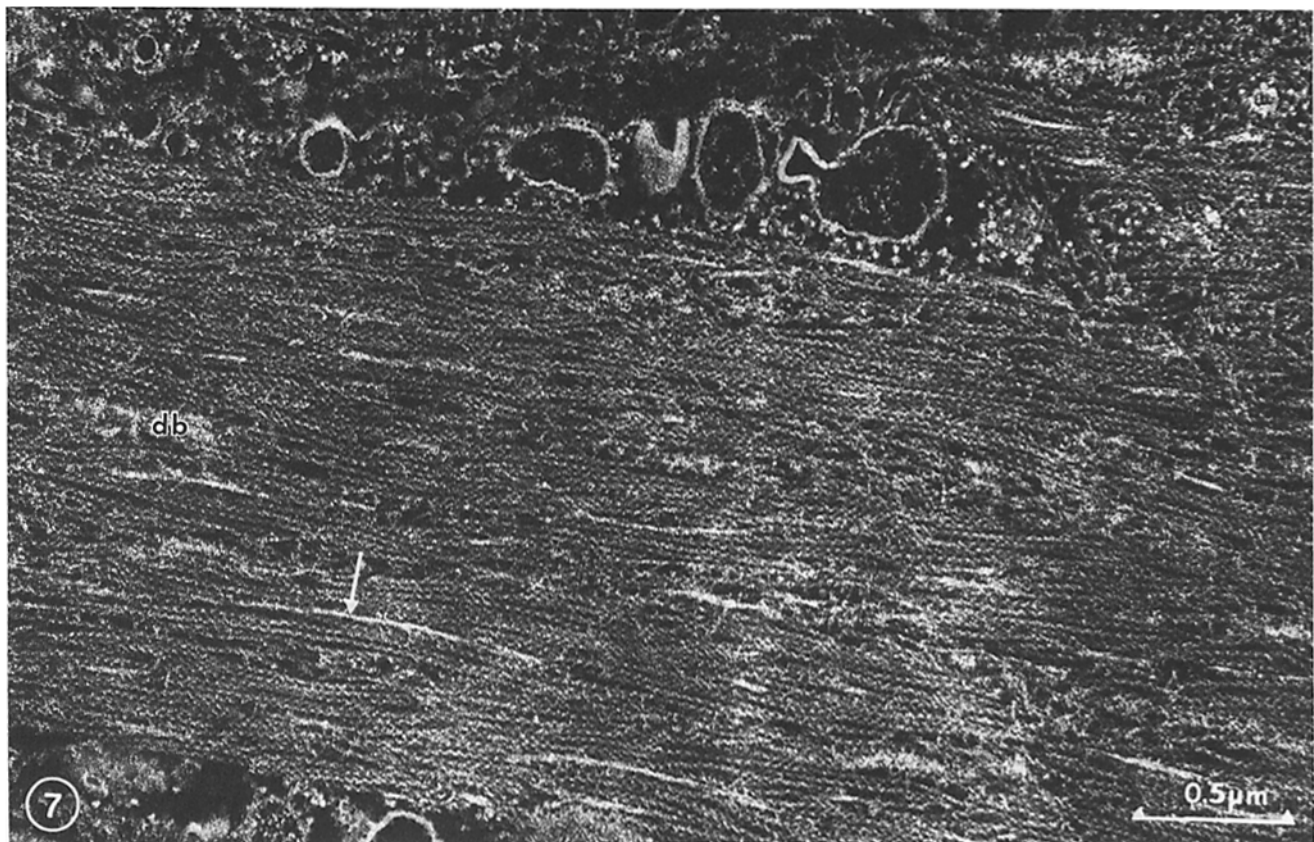

FIGURE 7 Low-magnification view of longitudinal section of a nonswollen saponin-treated smooth muscle cell from vas deferens in which the thin filaments are decorated with myosin S-1. The micrograph is reproduced with reversed contrast to better visualize the decorated filaments. Thick filaments (arrow) and cytoplasmic dense bodies (db) are shown. × 40,000.
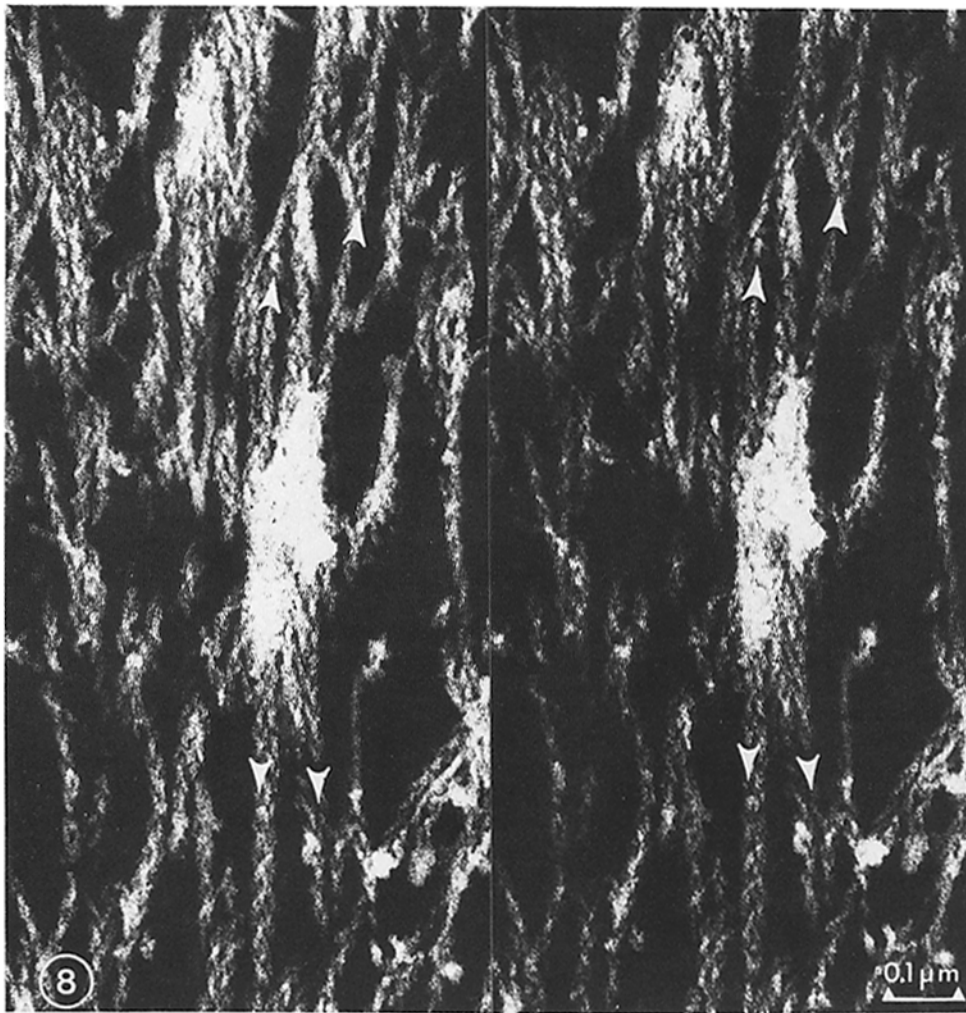
FIGURE 8 Stereo pair of a longitudinally sectioned cytoplasmic dense body from a saponin-treated vas deferens smooth muscle cell, swollen by incubation in the low ionic strength medium (solution A). The micrograph is reproduced with reverse contrast. S-1–decorated thin filaments (applied arrowhead markers) insert into each end of the dense body; the direction of the arrowheads resulting from S-1 decoration is away from the dense body at each end. Portions of 10-nm filaments are seen at the upper left of the dense body. × 95,000.
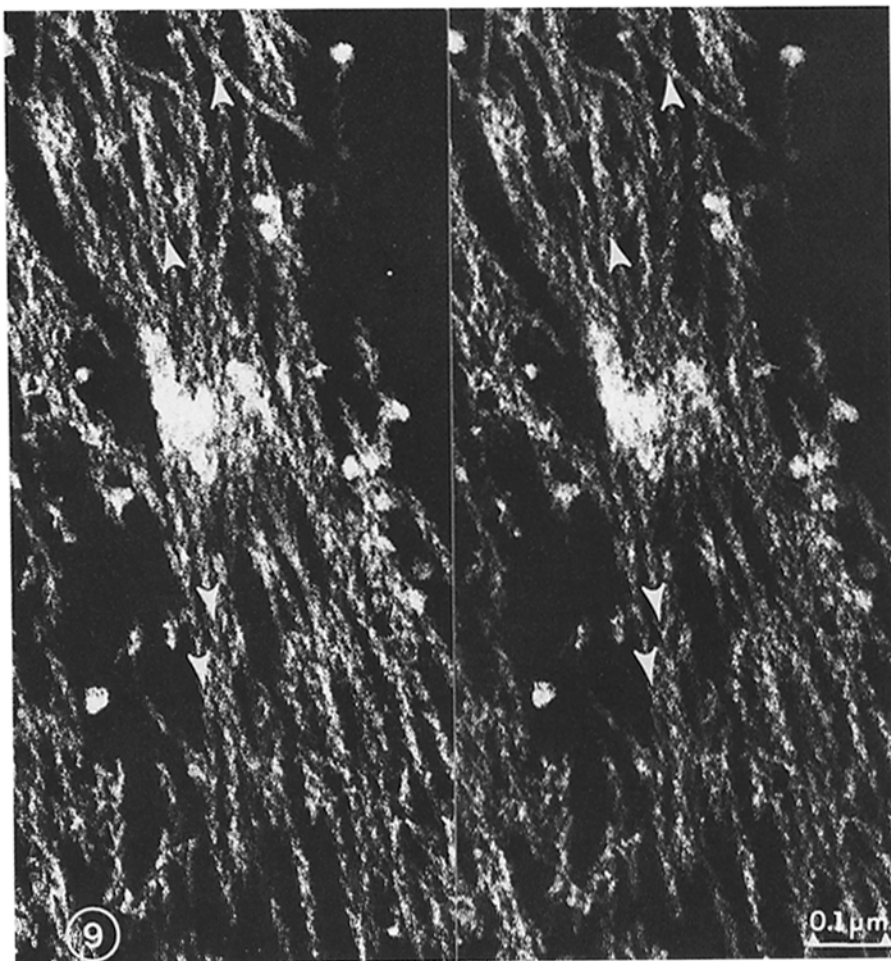
FIGURE 9 Stereo pair of a longitudinally oriented dense body from vas deferens smooth muscle which was processed in the same way as the cell shown in Fig. 5. S-1–decorated thin filaments insert into the dense body (applied arrowhead markers). The direction of the arrowheads from S-1 decoration is away from the dense body. × 95,000.
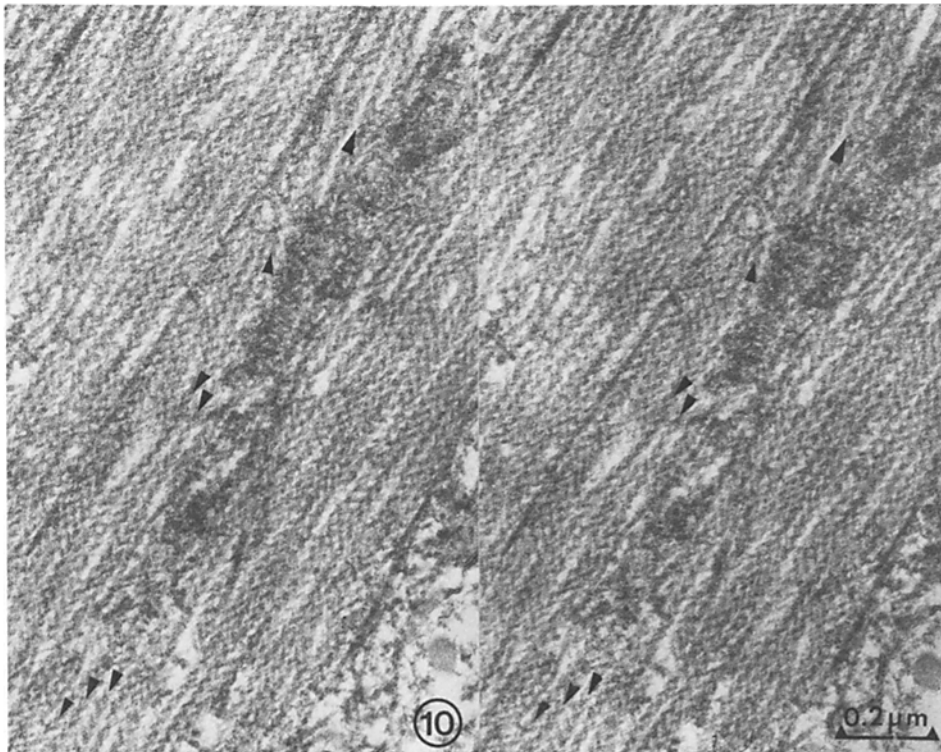
FIGURE 10 Stereo pair of a cytoplasmic dense body from a longitudinal section of vas deferens smooth muscle, saponin-skinned and incubated with S-1 in solution B (normal ionic strength). S-1-decorated thin filaments (arrowheads) insert into both the sides and ends of this long dense body. The applied arrowheads also indicate the polarity of the arrowheads formed by S-1 decoration. A thick filament is seen directly above and to the left of the dense body. × 60,000.
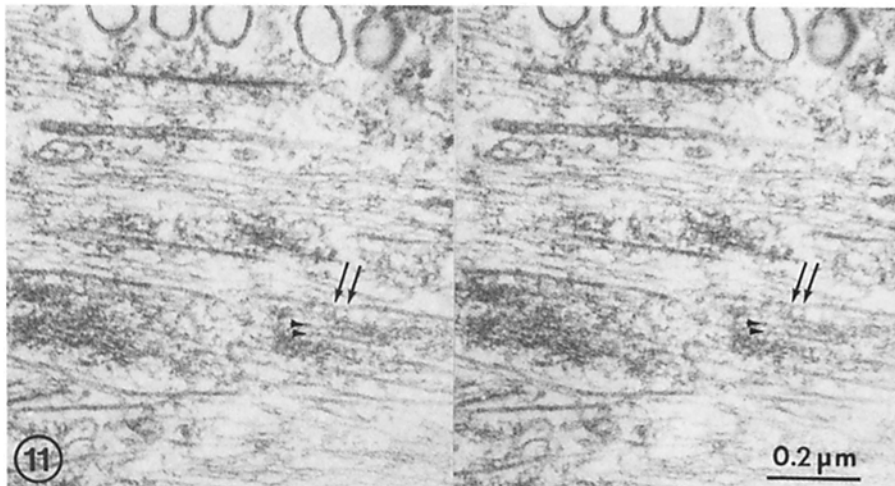

FIGURE 11 40-nm-thick longitudinal section of PAMV which was not treated with saponin or incubated in S-1. No tannic acid was added to the fixative. Arrows indicate an amorphous material that appears to connect the dense body with a 10-nm filament. Arrowheads point to thin filaments which lie within the matrix of the dense body. × 70,000.

the material connecting neighboring 10-nm filaments to the dense bodies (Figs. 2 and 11) could be α-actinin, which has been localized to the cytoplasmic dense bodies of smooth muscle (2, 15, 19, 44) and cross-links actin filaments in vitro (26). Recently, vinculin in addition to α-actinin has been localized at the surface, but not at the cytoplasmic dense bodies, of chicken gizzard smooth muscle cells (19).

Hubbard and Lazarides (22, 31) also presented evidence that smooth muscle actin and desmin can copolymerize in vitro, which may be related to their close association at dense bodies, and to how desmin (or vimentin) functions to link actin filaments in muscle cells (22).

The loss of myosin filaments that occurred in the swollen smooth muscle cells is a process that has been well documented in fixed preparations of nonskinned smooth muscle (27). In addition, the incubation of tissues at pH 7.1–7.2 may have resulted in increased solubilization of myosin; the solubility of myosin filaments in situ is known to increase with increasing

pH (29, 45) and maximal force development in skinned cells is also greater at pH 6.8 than at pH 7.2 (24). Proteolytic degradation of myosin may also have been taking place over the long incubation times (28).

In a previous study (1) in which 2.5–3.8 μm segments of three rabbit portal vein smooth muscle cells were reconstructed from 0.47 μm transverse sections, we observed that the 2.2 μm long myosin filaments occurred in groups of approximately five, with their tapered ends entering or leaving at the same level in the cell. We suggested that these groupings were minisarcomere units. Further evidence for an organization of thin filaments, thick filaments, and dense bodies into some type of contractile unit is provided by the present study which shows that the actin filaments attached to the dense bodies are of the right polarity (arrowheads pointing away from the dense bodies) for force generation with the groups of myosin filaments with which they are associated (Fig. 12). We have not pursued this idea further by attempting to measure "A-band" perio-

FIGURE 12 Longitudinal section of a saponin-skinned PAMV smooth muscle cell treated identically to the smooth muscle cell shown in Figs. 2 and 6. The filaments are splayed out in some of the cells, as shown, which reveals the relationship of the dense bodies with associated actin to the neighboring myosin filaments. The majority of the cells similarly treated show the more closely packed array of filaments as in Figs. 4 and 5. Thin filaments (indicated by arrows) which emerge from cytoplasmic dense bodies (db) can be traced to the myosin filaments on either side forming an I-band. The 10-nm filaments (arrowheads) do not run parallel to the sarcomeres. Connections between 10-nm filaments and a cytoplasmic dense body occur at the lower center part of the figure. × 70,000.

dicity, because of the difficulties inherent in following individual thin filaments from the dense body to the thick filaments in high magnification stereo views while at the same time obtaining low magnification views of thick sections of the same region to determine the periodicity. The 10-nm filaments which surround a given dense body do not run parallel to the contractile unit and associate with the next dense body in series, but are oriented obliquely toward another dense body of a different contractile unit. This linking of the dense bodies by 10-nm filaments in smooth muscle cells, although in a more oblique fashion, is reminiscent of the manner in which 10-nm filaments (desmin) are reported to interconnect myofibrils and the plasma membrane at the level of the Z-bands in skeletal muscle (30, 36, 38, 42) and at the Z-bands and intercalated disks of cardiac muscle (Fig. 3 in reference 7 and 17). However, it should be pointed out that there exists considerable controversy (21) as to whether some or all of the desmin observed by immunofluorescence (4, 30, 31) is present in a filamentous form in striated muscle.

We conclude that the cytoskeletal network of 10-nm filament connects the force generating units through their attachment to the Z-band-like dense bodies. This is not in agreement with the model proposed by others (8, 11, 46, 47) in which the dense bodies and 10-nm filaments are not integral components of the contractile units, but rather are part of a cytoskeletal network that is distinct and not connected to a contractile apparatus composed only of actin and myosin filaments. One of the arguments put forward for this model was that smooth muscle cells from which the 10-nm filaments had been removed through proteolysis still shortened in response to ATP (46). However, no information is given about the dense bodies in these extracted cells or about the magnitude of the force generated, its time course, reversibility, or mechanical properties. Without a comparison of the contractions of extracted cells versus intact cells, it is difficult to ascribe a role to the 10-nm filaments in force development. It is possible that they are involved in elastic recoil of the cell after shortening. In view of our findings of bipolar dense bodies, this shortening in the absence of 10-nm filaments implies that the contractile units and dense bodies are linked in series so that tension generated by the crossbridges can be transmitted throughout the cell.

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