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New views of smooth muscle structure using freezing, deep-etching and rotary shadowing

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Summary. Freezing, deep-etching and rotary shadowing techniques have been applied to study smooth muscle ultrastructure. The results show some new aspects of intracellular and extracellular connections, interior views of the sarcoplasmic reticulum showing a luminal content, coated pits and vesicles, contractile filaments and other organelles in smooth muscle.
Key words. Smooth muscle structure; freeze fracture.

Introduction

Electron microscopy has made significant contributions to our understanding of the mechanisms of excitation-contraction coupling and contraction in smooth muscle, through the identification of cellular organelles regulating cytoplasmic Ca and the visualization of filamentous contractile proteins (for reviews see Gabella[16], Somlyo et al.[46] and Somlyo[48]). However, the information conveyed by conventional electron micrographs, like by any other technique, is incomplete: it is also very much dependent on, and often distorted by, the preparatory techniques used[44]. For example, ultrathin sections provide a two-dimensional view that is generally insufficient for the understanding of the three-dimensional attachments and interrelationships of filamentous structures and membranes. In only rare instances[1] has three-dimensional information been presented in stereo electron micrographs of semithin sections of smooth muscle. Even more important, although conventional, liquid fixation with glutaraldehyde and osmium frequently preserves the in vivo structure, it is too slow for preserving rapid structural changes that occur in vivo. The combination of rapid freezing to preserve structure and, when necessary, subcellular composition on a millisecond time scale[23] with deep-etching and metal rotary shadowing of freeze-fractured tissues has recently been applied with great success to a variety of cell systems[20,22,23,25,57,58]. In some of these studies of static structures, stabilization with glutaraldehyde prior to freezing, as a matter of convenience, resulted in similar images to the non-fixed material. The replicas obtained in this manner afford a dramatic three-dimensional view of the fractured and etched surfaces along the cleavage plane and can often reveal with much greater clarity than conventional electron micrographs, the interrelationship of filaments and membranes. The images obtained from the replicas show surface topography whereas imaging of structures in conventional electron microscopy depends on their staining properties and differences in their density compared with that of the surrounding embedding medium. The structural information in the deep-etched rotary shadowed material can be further improved through the use of tissues permeabilized with saponin prior to freezing to remove the soluble proteins that obscure the view of filamentous proteins in replicas [22,25]. In such permeabilized preparations, the filamentous structures of interest can be further identified by reacting them with antibodies or other proteins. In this communication, we show the use of actin decoration with the S_1 subfragment of myosin as a means of identifying actin filaments and their polarity. The initial impetus for this study and its major objective was to determine the precise mode of the molecular assembly of myosin in smooth muscle, and is yet to be accomplished. However, we are able to present in this progress report some new aspects of intercellular connections and interior views of the sarcoplasmic reticulum, coated pits, filaments and other organelles in smooth muscle, and correlate these with the findings of conventional electron microscopy. We are encouraged to see that crossbridges that were initially demonstrated on myosin filaments in conventionally-fixed smooth muscle and described at a Discussion Meeting organized by Professor Bülbring[43] and subsequently shown in cryosections[45], can now be visualized in the three-dimensional view of these replicas.

Methods

Strips or sheets of smooth muscle were dissected from the rabbit portal anterior mesenteric vein (PAMV) or rabbit vas deferens, freed of connective tissue, stretched to approximately $1.7 \times L_0$ and incubated for approximately 1 h at 37 °C in oxygenated Krebs solution. The muscles were subsequently rinsed in relaxing solution and then incubated for 30–60 min at room temperature in 50–200 mg saponin/ml relaxing solution. The preparations in which actin was decorated with skeletal muscle myosin S_1 were saponin-skinned in a rigor solution, followed by incubation in 200 µg myosin S_1/ml for approximately 4 h at 4 °C prior to fixation. The relaxing solution contained 86 mM K-methanesulfonate, 10 mM EGTA (ethyleneglycol-bis-(β-aminoethylether)-N-N-tetracetic acid, 5.4 mM ATP (adenosine 5′-triphosphate-N′-disodium salt, 20 mM PIPES (piperazine-N,N′-bis 2-ethanesulfonic acid; 1,4-piperazinediethane sulfonic acid), 5 mM Mg-methanesulfonate. The rigor solution was the same as the relaxing solution, but without the ATP and with the addition of 10 mM phosphate buffer. Following skinning, the muscles were fixed in 2% glutaraldehyde with 0.075 M cacodylate buffer containing 4.5% sucrose. Prior to freezing, the fixed muscles were rinsed in 30% methanol for 5–10 min in order to wash out salts and to serve as a cryoprotectant. Methanol is volatile at the temperatures used for freeze-drying in the Balzer's apparatus. Small samples were mounted on brass holders, using 20% gelatin as an adhesive. The muscles were frozen in Freon 22, fractured at −110 °C, etched for 15 min at that temperature, rotary shadowed at −150 °C with platinum at 45°

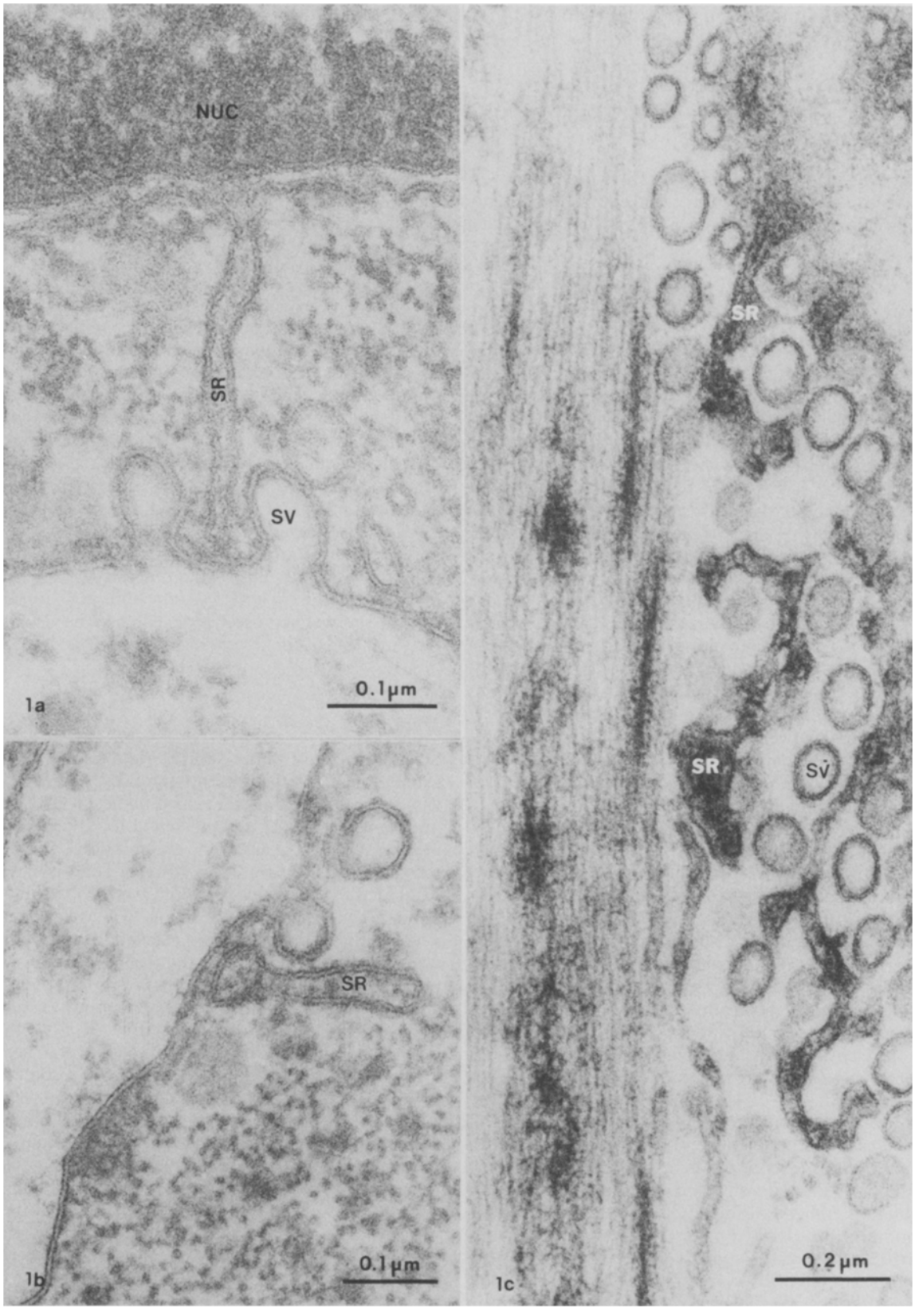


Figure 1. A portion of a transversely sectioned smooth muscle cell from the portal anterior mesenteric vein (PAMV) showing an element of sarcoplasmic reticulum (SR), the lumen of which is continuous with the perinuclear space is shown in figure 1a, and an SR surface coupling with an approximately 12–20 nm junctional gap between the SR and the plasma membrane in figure 1b. The reticulum has an osmiophilic staining material in its lumen. In stereo pairs (not shown) the amorphorus darkly staining material was shown to be within the lumen of the tubules in the elements of sarcoplasmic reticulum running between the tangentially sectioned surface vesicles in figure 1c. Glutaraldehyde fixation with 0.2% tannic acid. NUC, nucleus; SR, sarcoplasmic reticulum; SV, surface vesicle.

and replicated with carbon. The replicas were protected with collodion during cleaning with hypochlorite.
Examination was carried out in a Philips 400T or Zeiss 109 electron microscope operating at 80 kV. Stereo pairs were obtained with a high resolution goniometer stage tilted through ±7°. For illustration and study, the negatives were photographically reversed, and prints were made in negative contrast where the platinum-coated structures are highlighted against a dark background. This enhances the 3-dimensional appearance of the images, as originally suggested by Heuser and Kirschner[22]. Stereo pairs should be viewed with a pocket stereo viewer available from most electron microscopy units.

Conventionally-fixed preparations were prepared as described above up to the point of fixation in glutaraldehyde and tannic acid, followed by post-fixation in osmium, block staining in uranyl acetate, dehydration and embedding in Spurrs' resin[44].

Results and Discussion

Sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is a closed system of intracellular tubules occupying from approximately 2 to 7% of the cell volume in smooth muscle[11]. In occasional

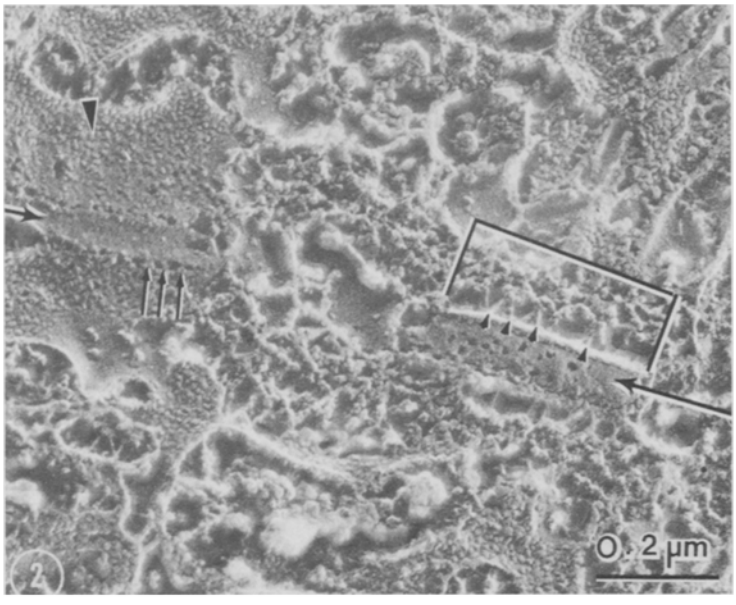


Figure 2. Longitudinal fracture at the level of the triad of toadfish swimbladder striated muscle. Deep-etched and rotary-shadowed. Large arrows point to the transverse tubules (tt). The tt on the left reveals its cytoplasmic leaflet and the one on the right, its luminal leaflet. The fracture has passed through the lumen of the terminal cisternae (TC), and shows a grainy meshwork of calsequestrin (boxed area) that is attached by strands (small arrowheads) to the TC membrane. Periodic feet crossing the junctional gap are indicated by small arrows. Where the SR membrane has split, Ca ATPase particles on the cytoplasmic leaflet of the SR can be seen (large arrowhead). (From a study by G. Nunzi and C. Franzini-Armstrong, reported at the 2nd int. Cell Biol. Congr., Berlin 1980.)

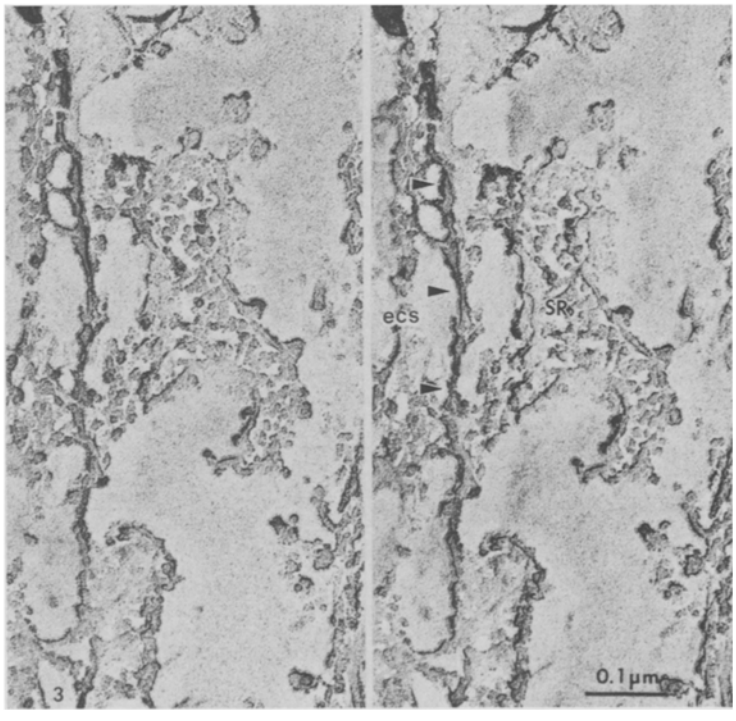
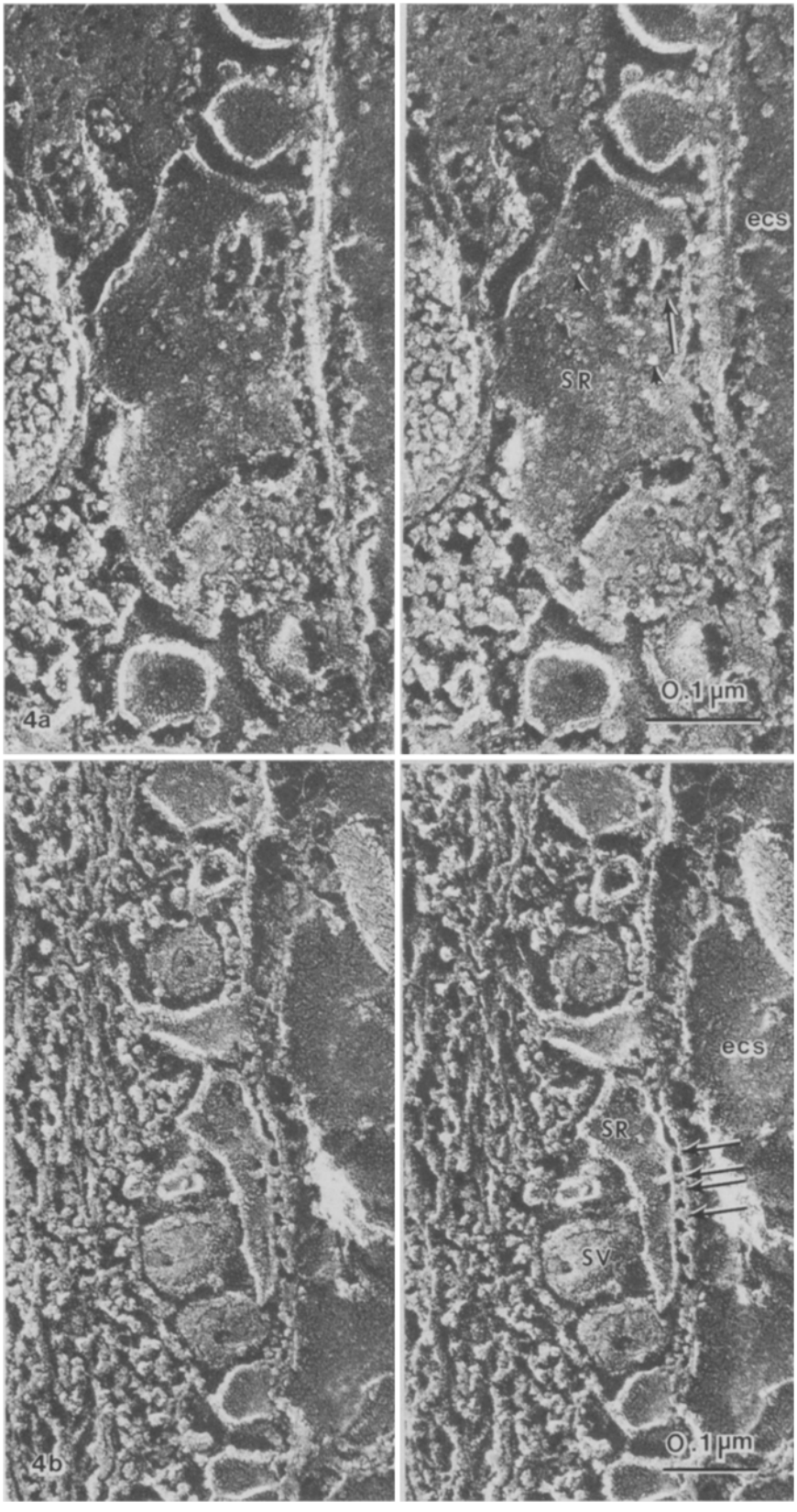


Figure 3. Stereo pair ±7° tilt of an element of junctional SR of rabbit PAMV where the fracture plane has passed through the lumen of the SR rather than following the SR membrane, revealing a granular content. The extracellular space is on the left hand side of the figure with the PAMV smooth muscle cell plasma membrane indicated by arrowheads. Glutaraldehyde-fixed with 0.2% tannic acid cryoprotected with 30% methanol, rapidly frozen, deep-etched and rotary shadowed. These negatives were not photographically reversed. ecs, extracellular space.



Figures 4a, b. Stereo pairs ±7° tilt showing two SR surface couplings in rabbit PAMV. In contrast to figure 3, the fracture plane has followed along the SR membranes, except for a small region shown by the arrow in 4a where strands of material within the lumen are connected to the SR membrane. Occasional intraluminal granules (arrowheads) have remained attached to the inner SR membrane. The extracellular space (ECS) is shown on the right. Periodic structures cross the gap between the surface membrane and the SR shown by arrows in 4b. Both the E and P faces of the surface vesicle membrane are exposed (labeled SV). Glutaraldehyde-fixed with 0.2% tannic acid, cryoprotected with 30% methanol, rapidly frozen, deep-etched and rotary shadowed.

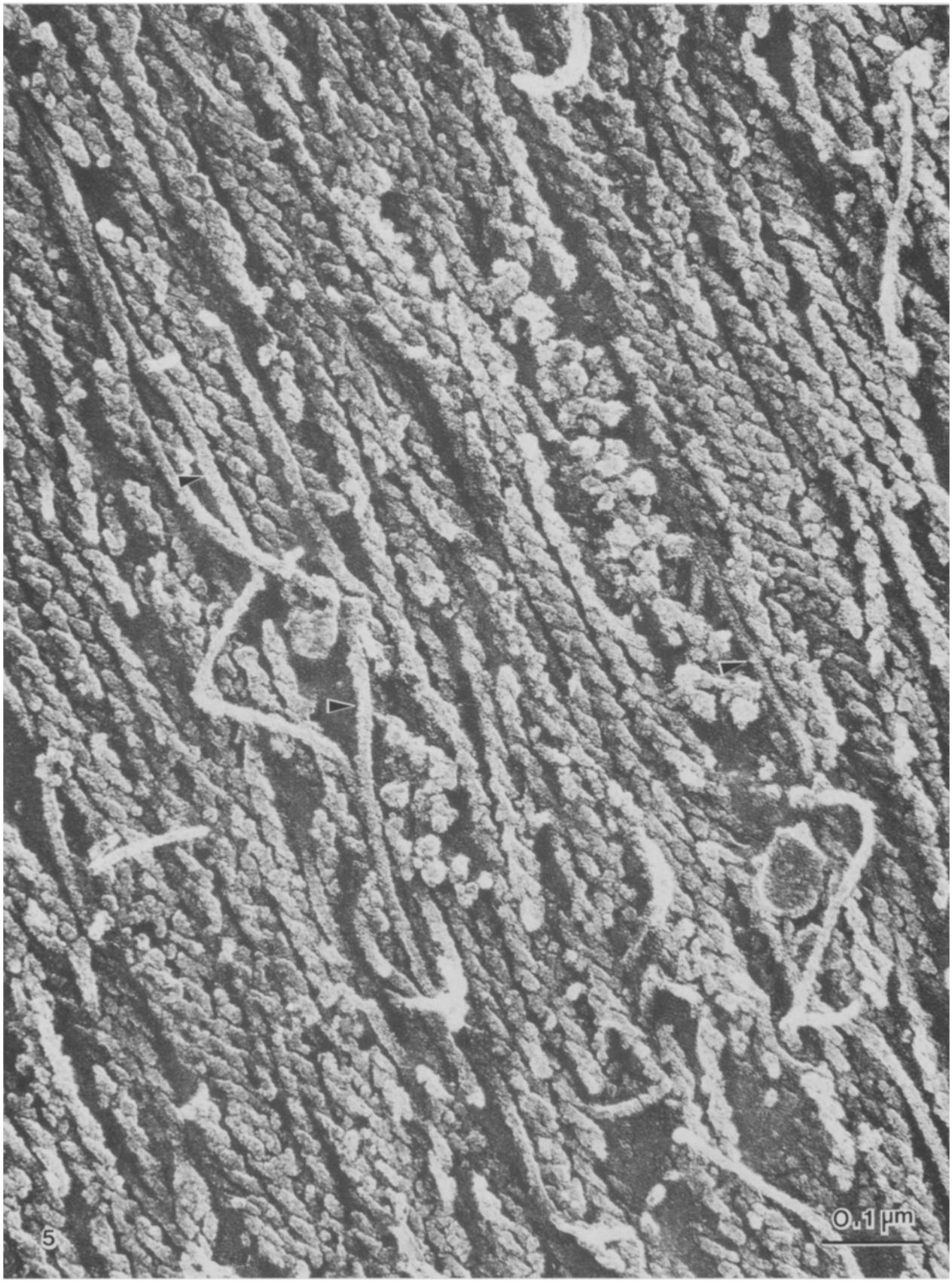


Figure 5. Longitudinal section of a saponin-skinned rabbit PAMV portion of a smooth muscle cell incubated with skeletal muscle myosin subfragment (S_1) prior to *fixation, freezing, deep-etching* and rotary shadowing. The rope-like structures are myosin S_1 decorated actin which shows a strong 38 nm half repeat. Smooth 10 nm filaments are indicated by arrowheads.

sections, a continuity between the lumen of the SR and the perinuclear space is revealed (fig. 1a in Somlyo and Somlyo[44]). The SR tubules at the periphery of the cell frequently establish close couplings with the surface membrane[11,42,47]; these structures are similar to the surface couplings in cardiac muscle[52]. In cryosections of rapidly frozen muscles, calcium has been directly measured in this peripheral SR[2]. Remarkably, even a small volume of reticulum (approximately 2% in the guinea pig portal vein) contains sufficient calcium to activate maximal contraction in the absence of extracellular Ca^{2+}, and this stored Ca can be internally recycled over relatively long periods of time in Ca-free solutions[2]. The calcium concentration measured in the junctional SR, about 28 mmoles/kg dry wt, may be an underestimate due to the larger diameter of the probe (75 nm) than the 30–40 nm diameter SR tubules and the use of cryosections that are thicker than the diameter of the SR. For comparison, the Ca concentration in the terminal cisternae (TC) of frog skeletal muscle is approximately 120 mmoles Ca/kg dry wt[50].

Calsequestrin, a low affinity, calcium binding protein has been localized to the TC of frog muscle[27,28] and appears as granular strands in conventionally-fixed and sectioned muscles. In rabbit and guinea pig portal vein smooth muscle fixed in the presence of tannic acid, a similarly staining material is frequently seen within the lumen of the junctional elements of the SR such as shown in figure 1.

In frozen, deep-etched rotary shadowed preparations, the presence of material within the lumen of the junctional SR was confirmed and, interestingly, resembled in part that seen in the TC of skeletal muscle prepared by the same techniques (fig. 2). The smooth muscle SR content has a globular appearance (fig. 3) and in some regions (arrow fig. 4a) the globular particles are attached to the wall of the SR by fine strands as observed in the skeletal muscle triad where the strands appear longer (fig. 2). It is possible, by analogy, that the material in smooth muscle SR is also a Ca-binding protein. In figures 4a and b the fracture plane followed along the membranes of the SR resulting in a smooth appearance, and

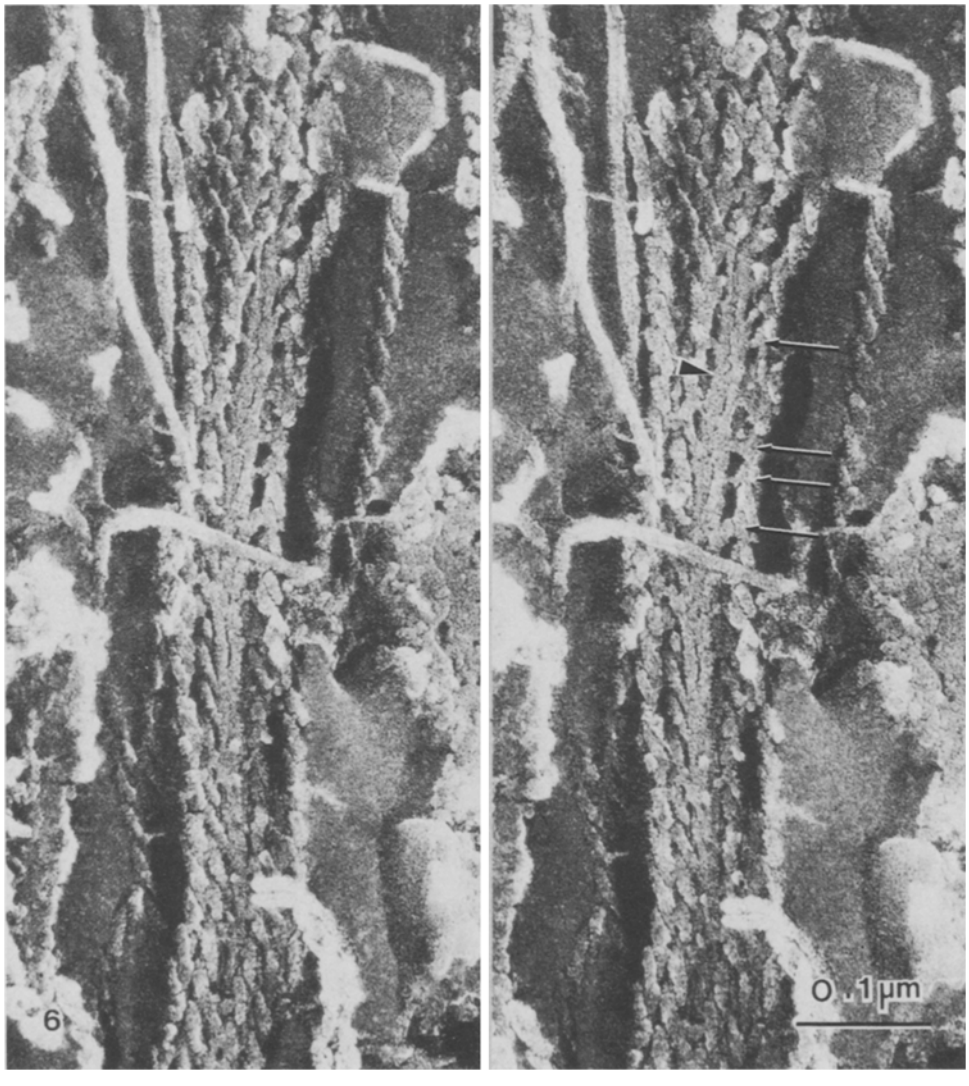


Figure 6. Stereo pair ±7° tilt of a frozen deep-etched rotary shadowed smooth muscle from the rabbit vas deferens showing a portion of a myosin filament (arrowhead) with crossbridges (arrows) attached to adjacent S_1 decorated actin filaments. The shaft of the filament when viewed in stereo appears to be cracked. Smooth, 10 nm filaments are also shown.

only in one region (3a, arrow) has it passed through the luminal content illustrating the necessity of the three-dimensional view offered by stereo pairs that can show that the material is actually within the lumen of the SR, rather than on the outer surface. The intraluminal SR content was present in saponin-skinned muscles incubated in EGTA containing solutions, and is, therefore, unlikely to consist of a precipitated form of Ca phosphate.

The junctional gap between the junctional SR and the plasma membrane at the close coupling sites is approximately 12–20 nm with periodic 'bridging structures' crossing the gap[11,42,47] resembling the surface couplings of cardiac[52] and the triads of skeletal muscle[13,14]. These structures appear as strands in the deep etched material (fig. 4b, arrows). In conventionally-fixed smooth muscle, the periodicity of the bridging structures is similar to the strands seen in the deep-etched specimens, and the spacing is somewhat less than the distance across the gap. In skeletal muscle the periodicity of the feet is approximately 29 nm and there appear to be two strands per foot. It is not known whether the bridging structures or feet play an active role in excitation-concentration coupling in muscle or whether they merely serve as mechanical struts maintaining the SR with its calcium stores close to the excitable surface membrane. It is highly unlikely that these feet or bridging structures provide an ionic communication with the extracellular space, such as found at gap junctions, because in situ quantitation of the elemental composition of the terminal cisternae of the SR of frog skeletal muscle with electron probe X-ray microanalysis[50,51] has shown that the Na, Cl concentrations are low and not different from the cytoplasmic concentrations. These findings rule out the suggestion[36] that the ionic composition of the SR is similar to that of the extracellular space.

Contractile apparatus: filaments and dense bodies

Regular arrays of myosin filaments are present in a large variety of smooth muscles in both the relaxed and contracted state[10]; for review see Campbell and Chamley[5], Gabella[16], Small and Sobieszek[41] and Somlyo[48]. Regular arrays of thick filaments have been demonstrated in unfixed, rapidly frozen and freeze-substituted muscles relaxed with isoproterenol, in which measurements on por-

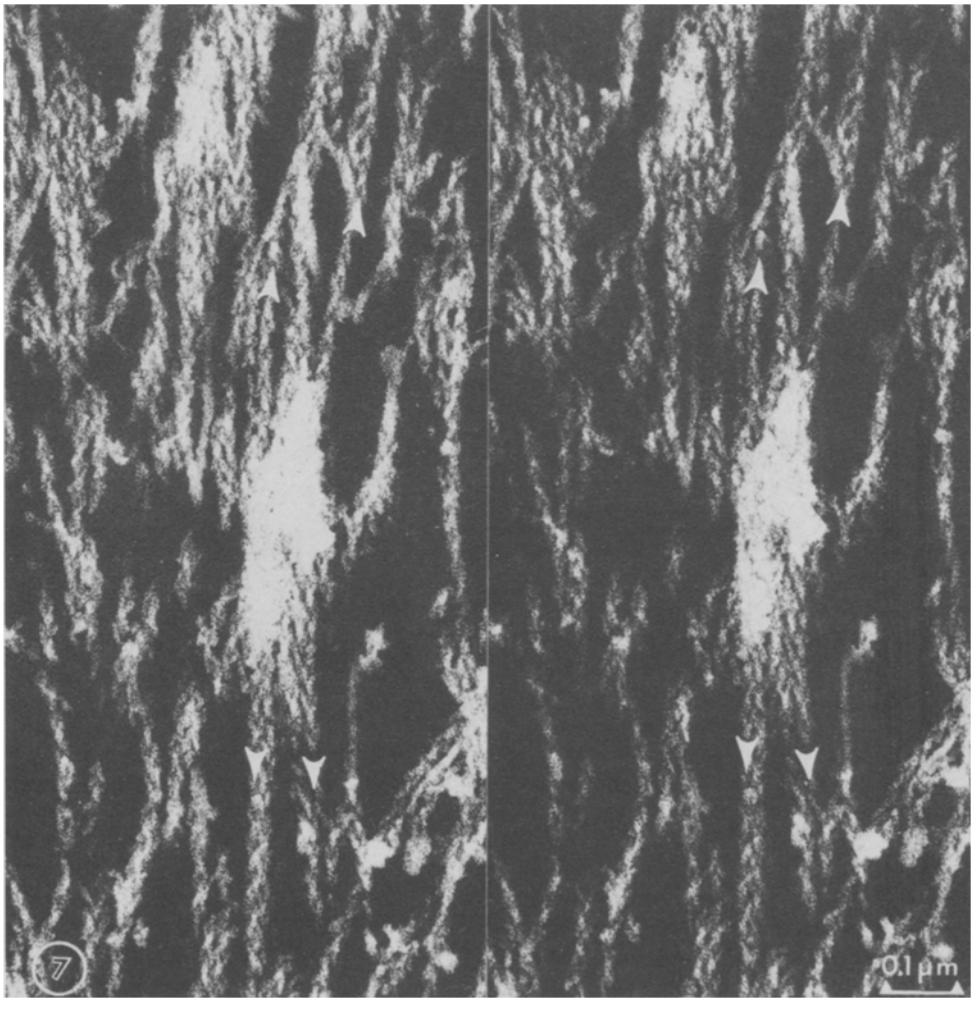


Figure 7. Stereo pair of a longitudinally sectioned cytoplasmic dense body from a conventionally-fixed, saponin-treated rabbit vas deferens smooth muscle cell. The micrograph is reproduced with reverse contrast. S_1 decorated thin filaments (applied arrowhead markers) insert into each end of the dense 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 in the upper left of the dense body. From Bond and Somlyo[3].

tions of the same tissue demonstrated that the 20,000 dalton light chains of myosin were not phosphorylated[49]. Therefore, light chain phosphorylation is not a necessary prerequisite for myosin to be in a filamentous form in smooth muscle, although the conformation of isolated myosin molecules can be modulated from the folded 10S to the straight rod 6S form by phosphorylation and/or low salt[9, 53, 54]. Recently, Trybus and Lowey[55] have reported that monomeric phosphorylated myosin can also form the bent 10S conformation.

The assembly of the myosin molecules in smooth muscle thick filaments in situ is not understood, although several models, based on the in vitro assembly of myosin molecules have been proposed. One of these is a conventional bipolar filament with a central bare zone, another a face polar filament with the molecules on one side arranged with the same polarity and opposite to the molecules on the other side[8].

We are currently trying to examine the in vivo myosin filament structure by using the deep-etching and rotary shadowing techniques[20, 21, 57, 58]. In preliminary experiments, muscles permeabilized with saponin[12] were incubated in a rigor solution to assure that crossbridges would be in an attached noncycling state. This was followed by decoration of the remaining available myosin-binding sites of actin with skeletal muscle myosin subfragment-1 (S_1). Decoration of actin filaments with myosin S_1 results in a rope-like structure, with prominent double helices having a half repeat of 38 nm, and allows clear identification of both the actin filaments and the 10 nm filaments that are not decorated with myosin S_1 (figs 5 and 6)[21, 22, 26]. A finer cross periodicity on each strand can occasionally be observed within the 38 nm half repeat and is consistent with the expected periodicity of myosin S_1 molecules attached to the underlying 7 actin monomers. The actin S_1 ropes appear to have a polarity[25], although it is not as obvious as the arrowheads seen in negatively stained material (fig. 7). The less apparent polarity in surface views than in negatively stained preparations was also observed by Wakabayashi and Toyoshima[59] in their three-dimensional reconstruction of the (striated muscle) actin-S_1 complex. We hope to determine the polarity of the in situ myosin filaments by identifying the polarity of the actin S-1 ropes that are attached by rigor crossbridges to neighboring myosin filaments, but we do not yet have a sufficient number of images in which

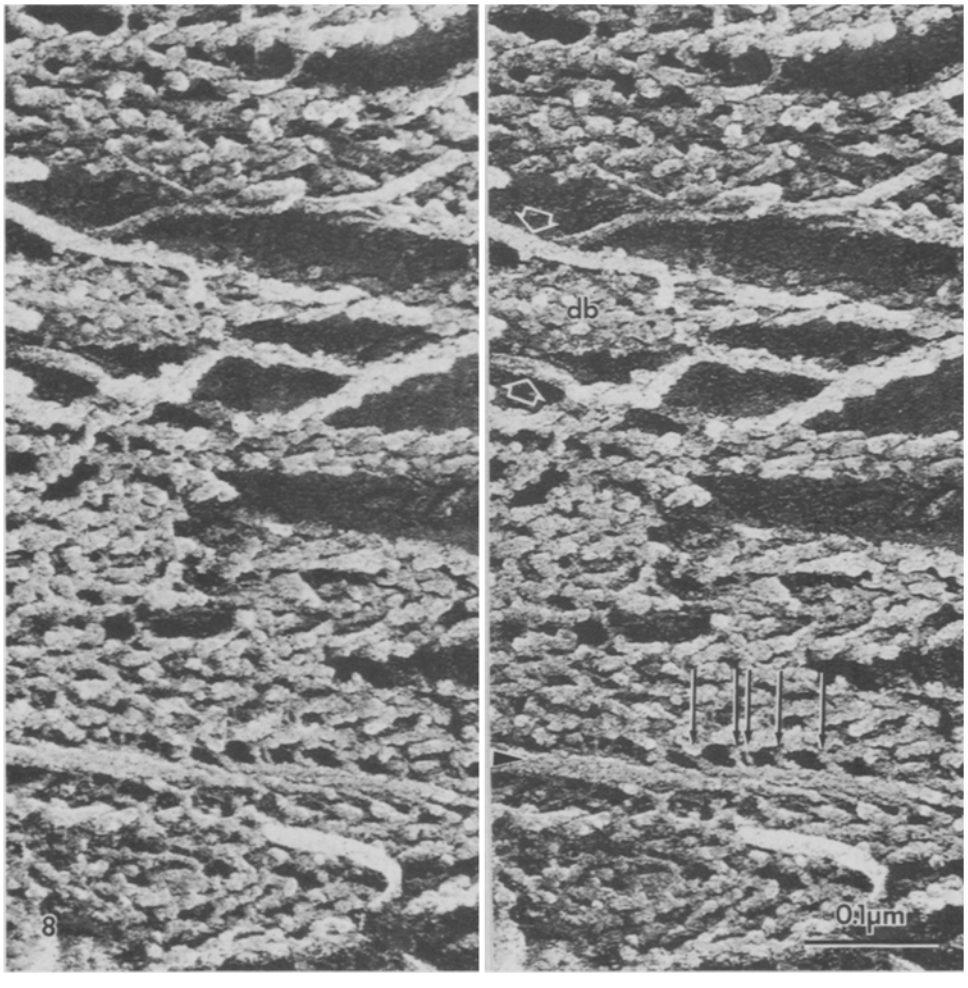


Figure 8. Stereo pair ±7° tilt of a portion of a smooth muscle cell from rabbit vas deferens and prepared in the same way as figure 6. A longitudinally oriented myosin filament (arrowhead) is shown running across the lower portion of the print with its crossbridges (arrows) attached to adjacent S_1 decorated actin. A portion of a cytoplasmic dense body (db) shown in the upper part of the print with its accompanying 10 nm filaments (white arrows) has a rather granular appearance. S_1 decorated actin appear to emerge from the right hand end of the db.

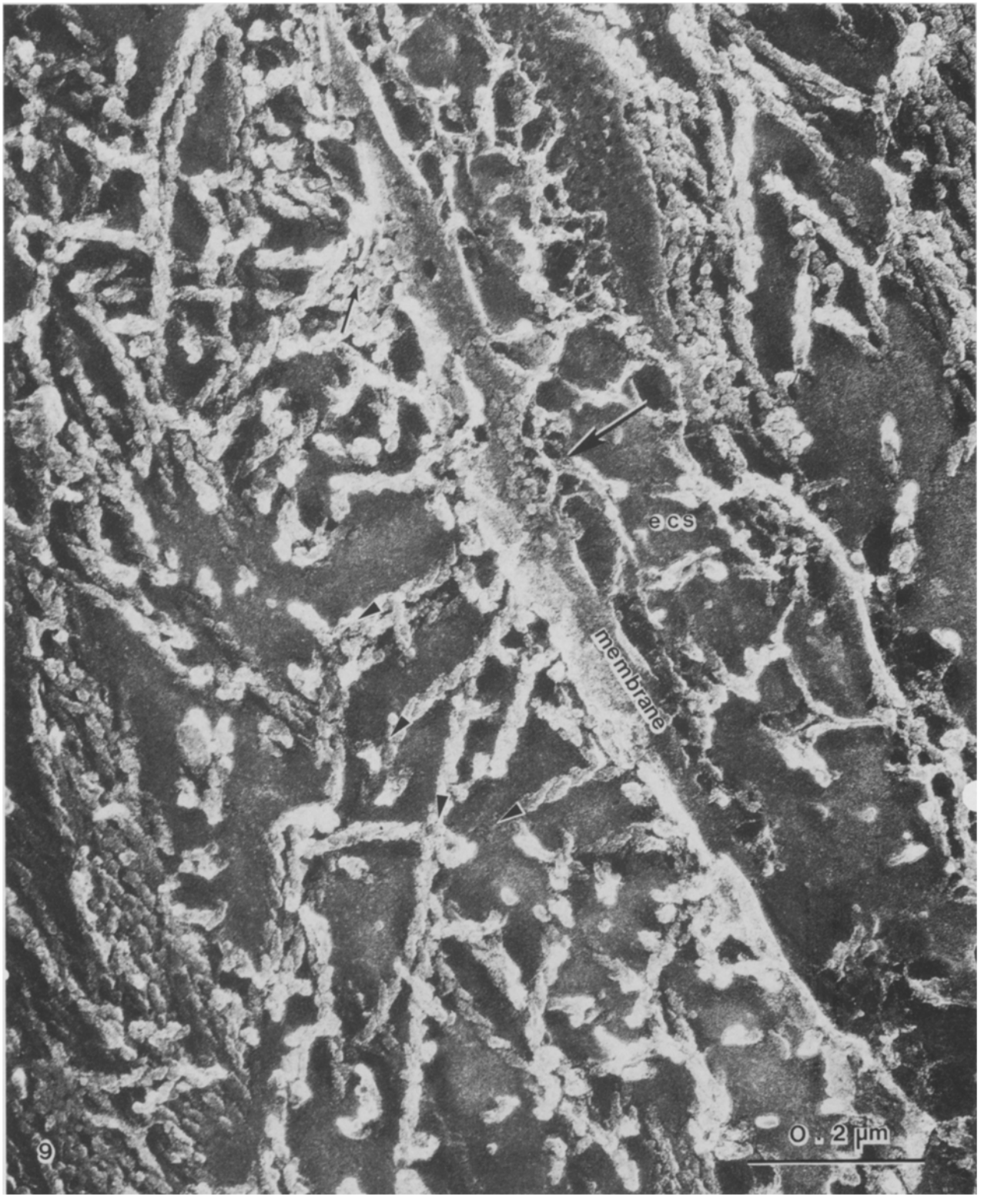


Figure 9. Longitudinal view of a portion of two cells and the intervening extracellular space (ecs) from rabbit vas deferens. The cell membrane is running diagonally and when viewed in stereo (not shown) comes toward the viewer as it curves to the left with the actin S_1 decorated filaments attaching at right angles to the membrane cytoplasmic face on the underside. The actin S_1 arrowheads point away from the membrane (arrowheads) and granular material is present in some regions where the filaments attach to the membrane (small arrow). Extracellular fibers terminate in granular material on the outer leaflet of the cell membrane (large arrow).

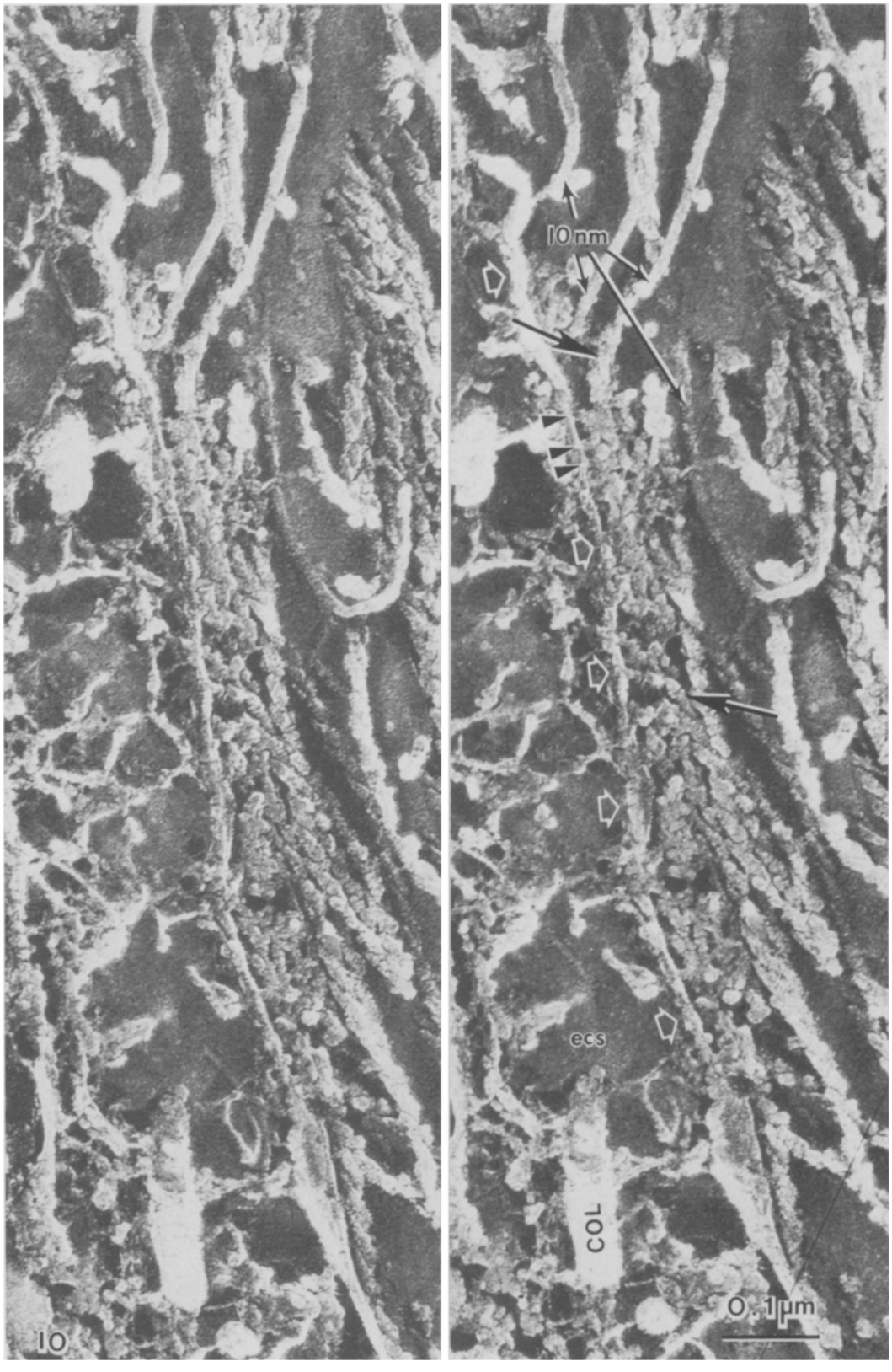


Figure 10. Stereo tilt pair ±7° from the same preparation as shown in figure 9 showing another cell membrane associated dense body where the inserting filaments lie parallel to the long axis of the cell. The extent of the dense body is indicated by the five open white arrows, the actin S_1 decorated filaments terminate in granular material (large arrows) which in turn appears attached to the cell membrane (arrowheads). 10 nm filaments (small arrows) are, in general, associated with smooth muscle dense bodies. The middle 10 nm filament shown at the top of the print has remained within the fracture plane and can be followed until it terminates in granular material at the cell membrane. The actin associated with the dense body is decorated with myosin-S_1 fairly close to its region of insertion on the membrane. Extracellular (ecs) fibrils insert on the outer aspect of the cell membrane and are attaching to collagen (COL) fibers.

the fracture plane has followed a sufficient length of the myosin filaments to reach a conclusion. The images, however, do show the periodic crossbridges previously observed in conventionally fixed smooth muscle, and the three-dimensional orientation of crossbridges is revealed in stereo pairs (figs 6 and 8)[1,43].

The dense bodies in the cytoplasm of smooth muscle react with antibodies to α-actinin[40], whereas vinculin has been localized only at the surface dense bodies attached to the plasma membrane[17]. At focal adhesion sites in fibroblasts, immunolabeled intracellularly for vinculin and α-actinin, vinculin labeling occurred closer to the membrane than α-actinin[7]. Dense bodies are equivalent to the Z-bands of striated muscles: as also shown by the polarity of the actin filaments inserting at these sites[3]. Arrowheads formed by the decoration of actin filaments with myosin S_1 can be seen, in stereo, to point away from either side of the dense body illustrated in conventionally

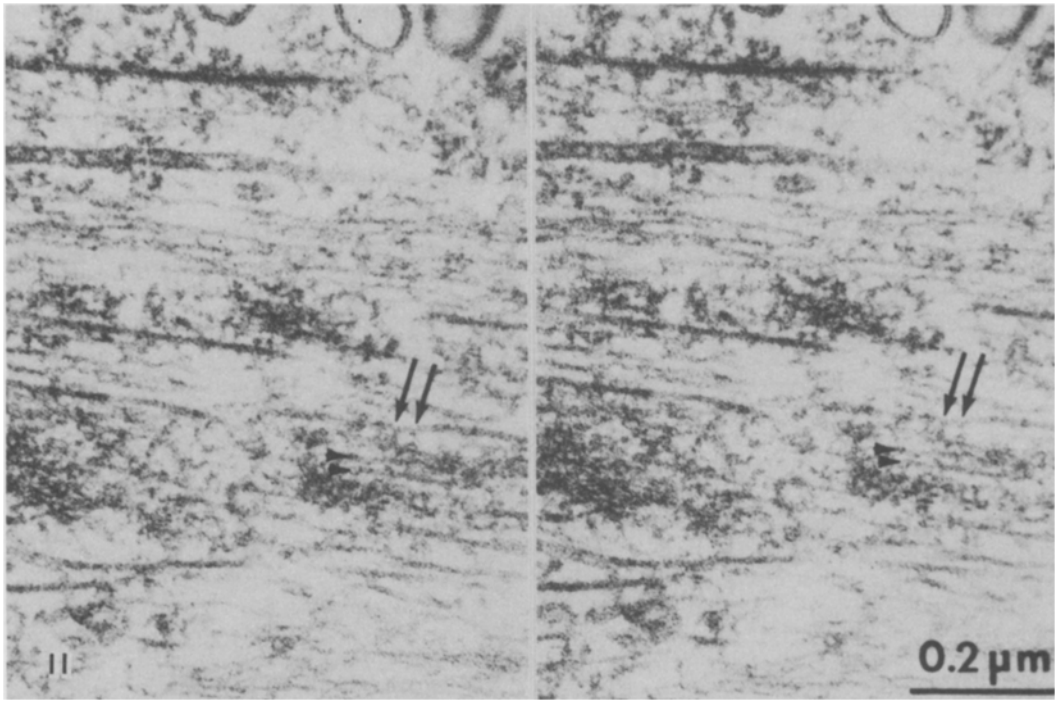


Figure 11. A thin (40 nm) longitudinal section of PAMV showing portions of two dense bodies. Arrows indicate strands that appear to connect the amorphous material of the dense body with a 10 nm filament. Arrowheads point to thin filaments which lie within the matrix of the dense body. From Bond and Somlyo[3].

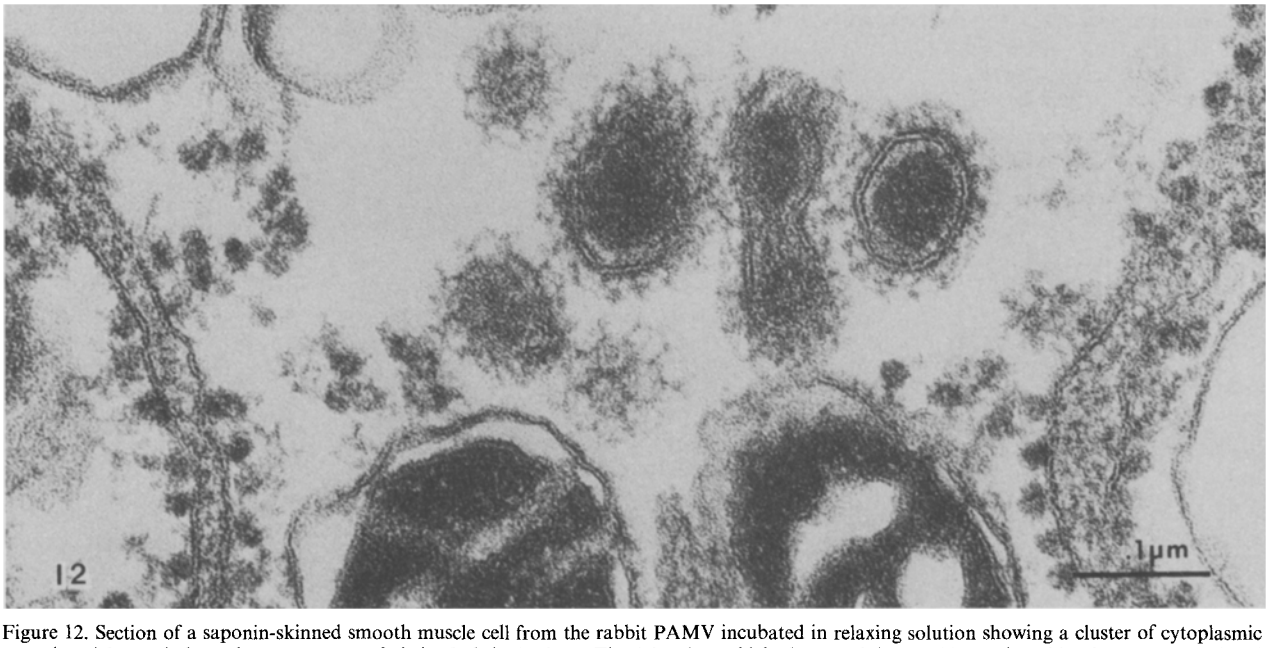


Figure 12. Section of a saponin-skinned smooth muscle cell from the rabbit PAMV incubated in relaxing solution showing a cluster of cytoplasmic coated vesicles and the polgon structure of their clathrin baskets. Fixed in glutaraldehyde containing 0.2% tannic acid prior to conventional post-fixation and plastic embedding.

fixed and stained sections in figure 7. The attachment of filaments to the surface membrane-bound dense bodies is also revealed in the saponin-skinned, frozen, deep-etched and rotary shadowed portal vein in figures 9 and 10. In figure 9, the S_1 decorated actin ropes are emerging on the left hand side of the membrane which, when viewed in stereo (not shown), appears to be coming towards the viewer and curving to the left. The connective tissue microfilaments with attachment granules are seen on the extracellular surface of the membrane, in a position suitable for force transmission to an adjacent cell. The filaments at this dense body are more spread out and are emerging at a much greater angle than typically seen (e.g. in fig. 10). Note that the filaments generally appear embedded in a granular material that appears attached to the membranes, and presumably consists of proteins such as vinculin and α-actinin (fig. 10). The middle 10 nm filament at the top of figure 10 can be followed to the cell membrane, where it terminates in granular material. In conventionally-fixed and stained preparations this dense body material has an osmiophilic and amorphous appearance, with occasional struts linking it to 10 nm filaments (fig. 11). Our interpretation of the actin polarity at these surface dense bodies is that the arrowheads point away from the membrane (arrowheads, fig. 9), as has been shown in conventionally-fixed and stained cells[3]. Hirokawa and Tilney[25] in a beautiful study of the actin filaments and their attachments in cochlear hair cells, have made a similar interpretation of S_1 decorated actin polarity in replicas, where they noted that the gyres of the 'ropes' were angular and assymetrical with a given gyre appearing fatter on one side than the other (see fig. 4 in Hirokawa and Tilney[25]).

Coated pits and coated vesicles

Coated pits and vesicles mediate absorptive receptor-mediated endocytosis in all nucleated animal cells. They sequester and transport widely different molecules bound to membrane receptors, for example, low density lipoproteins (LDL) (for review see Goldstein et al.[18]). Coated vesicles first described by Roth and Porter[38], are 50–300 nm in diameter and covered by a 20 nm bristle coat. Kanaseki and Kadota[29] were the first to analyze the 'vesicle in a basket' at the electron microscopic level, and proposed an icosahedral structure consisting of 8.5 nm walls or struts forming hexagons and pentagons which was subsequently described in more detail by Pearse[32,33] and by Heuser[19]. Isolation of coated vesicles from brain has shown that the baskets are composed predominantly of a 180,000 dalton polypeptide, clathrin[32,33], as well as three additional lower mol.wt polypeptides[56]. The polygon structure of coated vesicles in vascular smooth muscle cells is enhanced in thin sections of tissues fixed in the presence of tannic acid (fig. 12), and is shown very dramatically after rapid freezing, deep-etching, and ro-

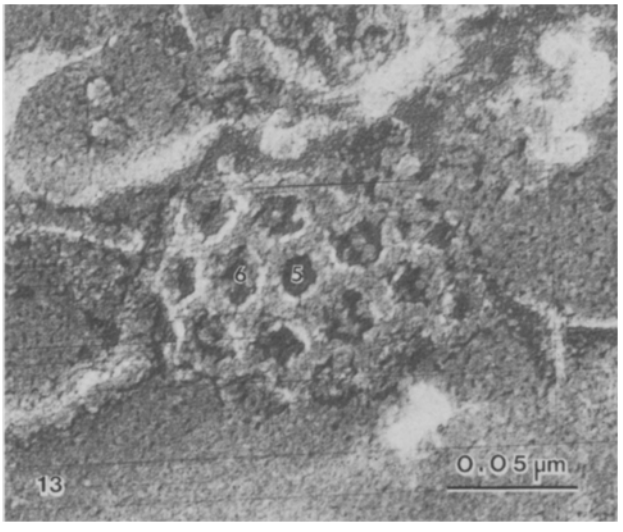


Figure 13. Frozen, deep-etched and rotary shadowed saponin-skinned smooth muscle cell incubated in rigor solution showing the polygon structure of a coated pit which is viewed from the inside of the cell with the cytoplasmic surface of the cell membrane seen in the background. Note the hexagon (6) and pentagon (5) formation of the clathrin basket.

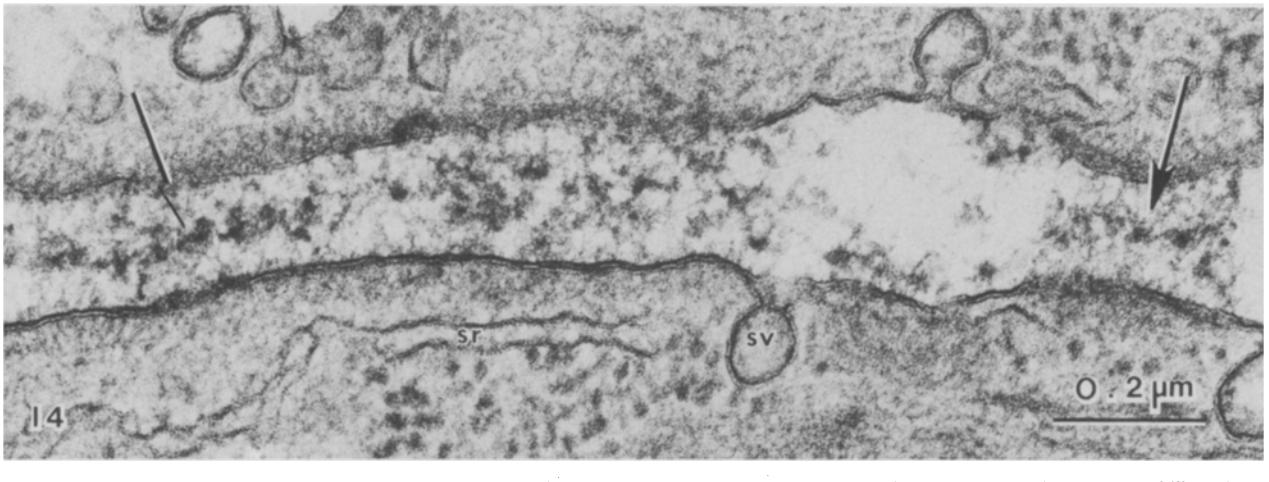


Figure 14. Longitudinal view of an extracellular attachment plaque (intermediate junction) between two rabbit smooth muscle cells. The midline where the basement membranes appear fused has osmiophilic staining granules (arrow). The faint strands attached to the membrane are more clearly seen in figure 15 after deep-etching and rotary shadowing. SR, sarcoplasmic reticulum, SV surface vesicle. Glutaraldehyde-fixed with 0.2% tannic acid followed by conventional post fixation and embedding.

tary shadowing (fig. 13): the stereo views are similar to the images of coated vesicles in fibroblasts shown by Heuser[19]. The hexagonal and pentagonal construction of the clathrin baskets of smooth muscle coated vesicles can be seen in figure 13. The presence of coated vesicles in rabbit portal vein was more apparent in saponin-skinned preparations: this could merely reflect a better preservation of the structures in the hyperpermeable cells or could reflect a real, saponin-induced increase in endocytosis. Coated vesicle membranes are low in cholesterol[30] and, therefore, while saponin causes clustering of cholesterol molecules within the plasma membrane and subsequent breakage[31], it should not disrupt the coated pits and coated vesicles. During the early phases of 'skinning' the loss of unesterified cholesterol from the cytoplasm would be expected to increase the number of LDL receptors on the cell membrane, and this could stimulate the LDL receptor mediated endocytosis pathway described by Goldstein et al.[18]. These authors have also shown that the clustering of the LDL receptors in coated pits, internalization and recycling can occur whether or not LDL is present. Interestingly, cytoplasmic calmodulin is redistributed to regions under the membrane receptor clusters or ligand capped areas in human lymphoblastoid cells. This calmodulin redistribution is calcium dependent and endocytosis is blocked by trifluoperazine[39].

Extracellular matrix

The extracellular matrix of blood vessels consists of collagen and elastin fibers embedded in a ground substance containing mainly glycoproteins and proteoglycans.

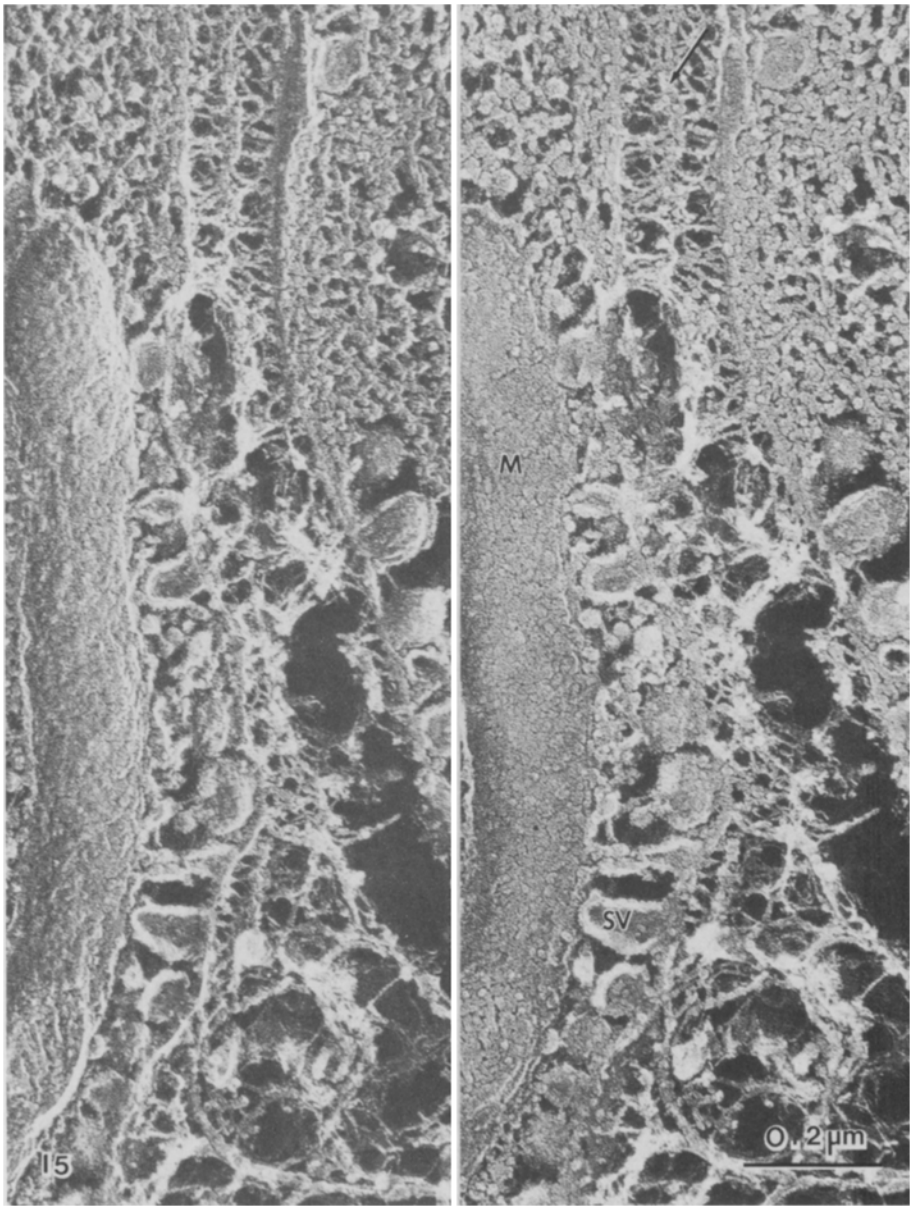


Figure 15. Stereo pair ±7% from rabbit PAMV showing a similar intermediate junction or attachment plaque (arrow) as shown in figure 14 but tissue prepared by deep-etching and rotary shadowing. The basement membrane fibrils are attached to the cell membrane at right angles. M, mitochondria; SV, surface vesicle.

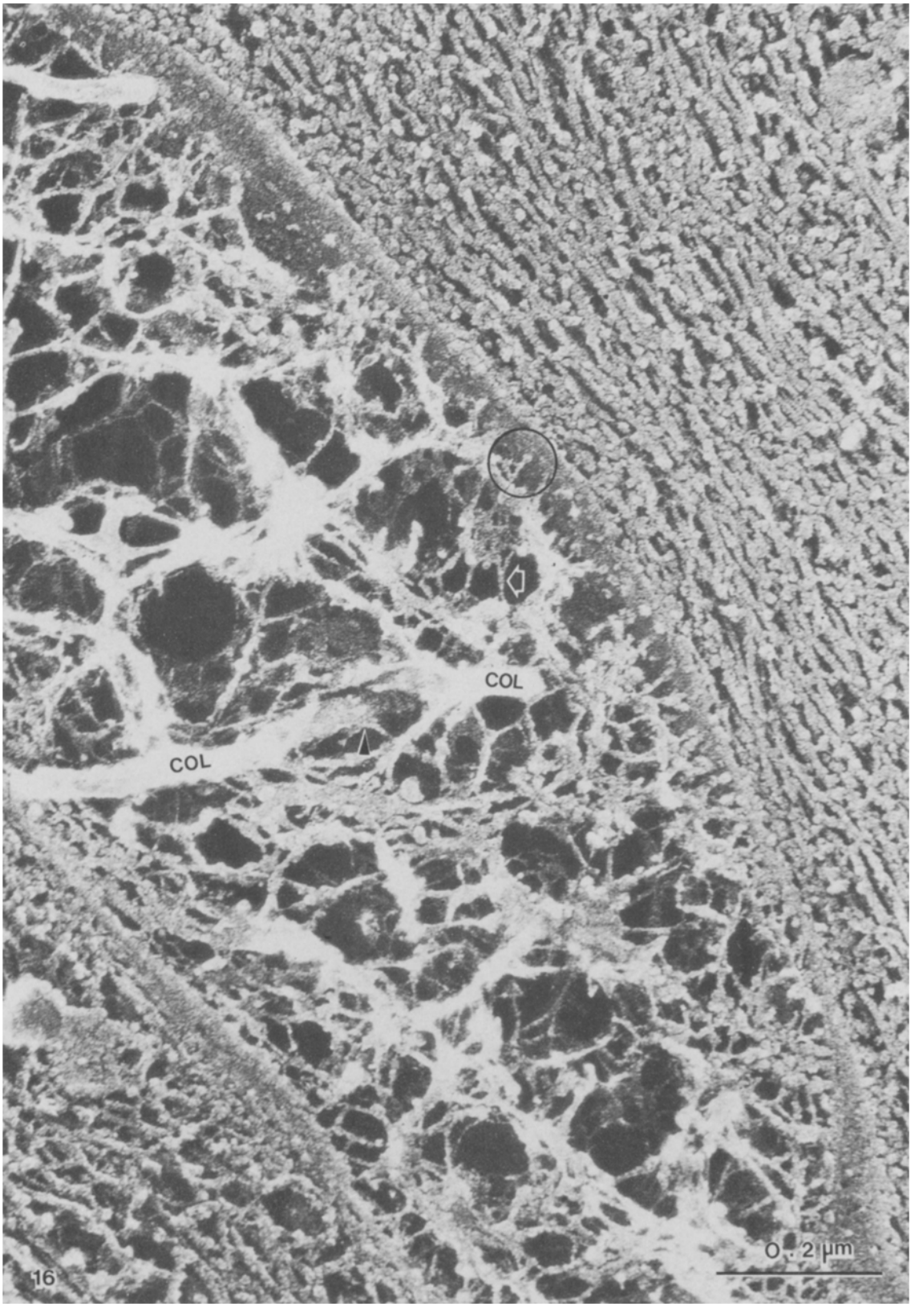


Figure 16. A view of the extracellular matrix between two smooth muscle cells of the rabbit PAMV showing a dense network of fibrils (open white arrow) which in turn are attached to the basement membrane fibrils which frequently terminate with a granule on the outer leaflet of the plasma membrane (encircled region). Fibrils wrap around and are attached to the collagen (COL) fibers (black arrowhead). Note the granular material of varying size attached to the fibrilar network. Prefixed, cryoprotected with 30% methanol, frozen, deep-etched and rotary shadowed.

Smooth muscle cells have been shown to synthesize the connective tissue macromolecules of the arterial wall (for review see Burke and Ross[4] and Ross[37]). Proteoglycans are macromolecules consisting of carbohydrate polymers (glycosaminoglycans) that are covalently bound to a protein core and serve as structural links between the elastin and collagen fibers and the cells. A similar matrix has been described in cardiac tissue[35]. The strongly anionic proteoglycans appear to be readily lost during tissue preparation for electron microscopy, unless they are first precipitated with cationic dyes such as ruthenium red[61]. The staining of extracellular granules and filaments by ruthenium red and other cationic dyes, as well as their solubilization by chondroitinases or hyaluronidase, is considered diagnostic for glycosaminoglycans. Wight and Ross[61] observed 20–50 nm ruthenium red staining granules with filamentous projections dispersed between and attached to collagen and elastin fibers, and a smaller class of ruthenium red positive granules (10–20 nm diameter) within the basement membrane of smooth muscle cells in primate arteries.

The use of tannic acid during fixation dramatically enhances the affinity of the extracellular matrix for the subsequent stains, as shown in figure 14. This high magnification view shows a region between two smooth muscle cells, where their basement membranes appear to be fused and the densely staining granules along the midline appear similar to the ruthenium red staining granules described by Wight and Ross[61]. These structures, called attachment plaques or intermediate junctions[15,16], are more clearly defined in prefixed rapidly frozen, deep-etched and rotary shadowed material, as shown in stereo in figure 16, where the basement membranes are composed of fibrils attached to and oriented at right angles to the cell membrane. The midline also appears fused, but contains fewer granular type structures than seen in the fixed and embedded material. The diameter of these basement membrane fibrils is roughly half the diameter of the actin filaments in the same metal replica. The insertion of the basement membrane fibrils on the outer surface of the cell membrane is readily demonstrated in the deep-etched material (figs 15 and 16), where the fibrils frequently terminate on the membrane with a globular particle (circle in fig. 16).The deep-etched material also reveals an extensive web-like network of fibrils attached to collagen fibers and extensively connecting neighboring cells. There are granules of varying sizes attached to this web of fibrils. This network is dramatically more pronounced in the deep-etched than in conventionally-fixed and embedded rabbit portal vein, and differs from that seen in primate arteries treated with ruthenium red to precipitate proteoglycans; in the latter preparation many more granules and fewer fibrils are preserved[61]. Chen and Wight[6] have shown that a 40% loss of radioactive sulfated glycosaminoglycans occurs during routine processing of tissues for plastic embedding for electron microscopy, and 84% of this total loss takes place during post-fixation and washing of the tissue (in the absence of ruthenium red treatment). In the extracellular matrix of both vascular tissue and of cartilage, the proteoglycans collapse due to dye-binding, dehydration and fixation[34,60]. The deep-etched material illustrated has been prefixed with glutaraldehyde and cryoprotected with 30% methanol prior to freezing, but has not been post-fixed, block stained and completely dehydrated such as the plastic embedded material, and this may account for the much lower density of granules and the more extensive fibrillar network. Antibodies to proteoglycan monomers and to the link protein have greatedly aided the definition of the organization of bovine articular cartilage[34], however, the interpretation of this data is complicated by the suggested proteoglycan collapse during routine fixation and plastic embedding. If the rapid freezing and deep-etched preparations represent the natural organization of the extracellular matrix, then this technique used in conjunction with digestive enzymes and/or antibodies to the constituents of the extracellular matrix, should offer a very useful approach and aid in the understanding of the interactions of these matrix macromolecules in both normal and diseased state. Antibody localization, without the use of ferritin or collodial gold tags, with the quick freeze, deep-etching technique has recently been shown to be feasible, and was used to localize neurofilament polypeptides in the neuronal cytoskeleton[24].

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