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Sarcoplasmic reticulum and mitochondria as cation accumulation sites in smooth muscle

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[Plates 9 to 11]

A tubular system of sarcoplasmic reticulum that is not penetrated by extracellular markers is described in vertebrate smooth muscles. The sarcoplasmic reticulum forms fenestrations around the surface vesicles and also forms close appositions (an approximately 10 to 12 nm gap traversed by periodic electron dense material) with the non-specialized surface membrane. The morphological couplings are considered to be the most probable sites of electromechanical coupling of the action potential to the twitch contraction. The relative volume of the sarcoplasmic reticulum varies in functionally different (tonic and phasic) smooth muscles, and correlates with the ability of the different smooth muscles to contract in the absence of extracellular calcium. Electron opaque deposits of strontium are accumulated by peripheral and central elements of the sarcoplasmic reticulum. The accumulation of strontium and barium by mitochondria raises the possibility that, in addition to the sarcoplasmic reticulum, mitochondria may play a role in the regulation of intracellular divalent cation levels in vertebrate smooth muscle.

Contraction of smooth muscle may be triggered by an action potential (Bozler 1948; Bülbring 1955; for other references see Bülbring, Brading, Jones & Tomita 1970; Johansson 1971), by graded depolarization elicited by excitatory agents (Su & Bevan 1965; Somlyo & Somlyo 1968*a*; Somlyo, Vinall & Somlyo 1969; Haeusler 1972), or by a process (pharmacomechanical coupling) that is not directly controlled by a change in membrane potential (Evans, Schild & Thesleff 1958; Somlyo & Somlyo 1968*a*; for other references see Somlyo & Somlyo 1968*b*, 1970). Although in some smooth muscles the magnitude of the maximal contractile response to different drugs is proportional to the magnitude of depolarization produced (Somlyo *et al.* 1969), smooth muscles also contract even when the membrane is depolarized by potassium ions in virtually calcium-free solutions (Bozler 1969; Somlyo *et al.* 1971; Devine, Somlyo & Somlyo 1972). Clearly, such drug-induced contractions in Ca-free media, while perhaps indirectly controlled by changes in the ion permeability of the membrane, are not directly activated by an inward flux of extracellular calcium. Similarly certain twitch contractions of mammalian (Bülbring & Tomita 1970) and reptilian smooth muscle (Somlyo, Devine, Somlyo & North 1971) in virtually Ca-free solutions must be activated by calcium from some source other than the extracellular fluid.

The above physiological observations raised the question whether there is, in vertebrate smooth muscles, a sarcoplasmic reticulum that can accumulate and release divalent cations as in striated muscles (Peachey 1968; for other references see Bianchi 1968; Huxley 1971). It was necessary, therefore, to estimate the approximate volume of the sarcoplasmic reticulum-like tubules already noted by earlier authors (Caesar, Edwards & Ruska 1957; for other references see Somlyo & Somlyo 1968*b*; Burnstock 1970), and to establish that these structures constitute a true sarcoplasmic reticulum that is not in direct communication with the extracellular space, but forms sufficiently close contacts with the surface membrane to allow electromechanical

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coupling and the release of calcium from the sarcoplasmic reticulum by the action potential. In view of the marked differences between the sarcoplasmic reticulum of phasic and tonic striated muscles (Huxley 1964), it also appeared of interest to compare the ultrastructure of the spike generating, relatively phasic, smooth muscles such as the portal-anterior mesenteric vein and taenia coli, with that of the gradedly responsive, tonic smooth muscles of some large elastic arteries (Somlyo & Somlyo 1968*a*).

Methods

The preparatory techniques for electron microscopy have been described elsewhere (Somlyo *et al.* 1971; Somlyo & Somlyo 1971*a*; Devine *et al.* 1972; Somlyo *et al.* 1973; Somlyo & Somlyo, in press).

Results and discussion

Examination of smooth muscles incubated with extracellular markers prior to (ferritin) or after (colloidal lanthanum) fixation showed that the extracellular markers do not enter the structures normally identified as the sarcoplasmic reticulum (figure 1, plate 9) in smooth muscle (Somlyo *et al.* 1971; Devine *et al.* 1972). The sarcoplasmic reticulum consists of a three-dimensional tubular network with the majority of the tubules having a predominantly longitudinal orientation. Tangential sections of the sarcoplasmic reticulum of turtle smooth muscle also include larger sheets of membrane that are continuous with the tubules (figure 8 in Somlyo *et al.* 1971). Peripheral elements of the sarcoplasmic reticulum adjacent to the plasma membrane are in direct communication with the more centrally located elements through radially oriented tubules (Somlyo *et al.* 1971; Devine *et al.* 1972). In longitudinal sections of smooth muscle (figures 2, 3, plate 9) a particularly common type of arrangement is the network of sarcoplasmic reticulum fenestrated around the surface vesicles (Somlyo *et al.* 1971; Gabella 1971; Devine *et al.* 1972) observed in every type of vertebrate smooth muscle that we have thus far examined. Such groupings of sarcoplasmic reticulum–surface vesicle complexes are separated by intervening dense bodies (figure 5, plate 10). The separation of groups of surface vesicles by dense bodies has been suggested from examination of freeze-etched vascular smooth muscle (Devine, Simpson & Bertaud 1971).

The volume of the sarcoplasmic reticulum varies in the functionally different smooth muscles. We have found a particularly large volume of sarcoplasmic reticulum (approximately 5 % of cell volume, uncorrected for tangential sections) in the smooth muscle of mammalian large elastic arteries (figure 6, plate 10), an intermediate (approximately 3 %, uncorrected) amount (and rather variable among different animals) in the oviduct of the turtle, and the smallest amount (approximately 2 %, uncorrected) in some of the spontaneously active spike-generating mammalian smooth muscles (e.g. portal-anterior mesenteric vein and taenia coli). The smooth muscles that contract even after prolonged exposure to calcium-free solutions also have a larger volume of sarcoplasmic reticulum and much of this tends to be relatively centrally located (Somlyo *et al.* 1971; Devine *et al.* 1972). This correlation between functional and structural properties, while seemingly direct, may however be fortuitous.

The larger volume of sarcoplasmic reticulum in the gradedly responsive, tonic smooth muscles than in the spike generating, phasic smooth muscles, contrasts with the inverse correlation found in striated muscles (Huxley 1964). It is unlikely, though, that the surface membrane properties and the volume of sarcoplasmic reticulum of smooth muscle are related in the simple

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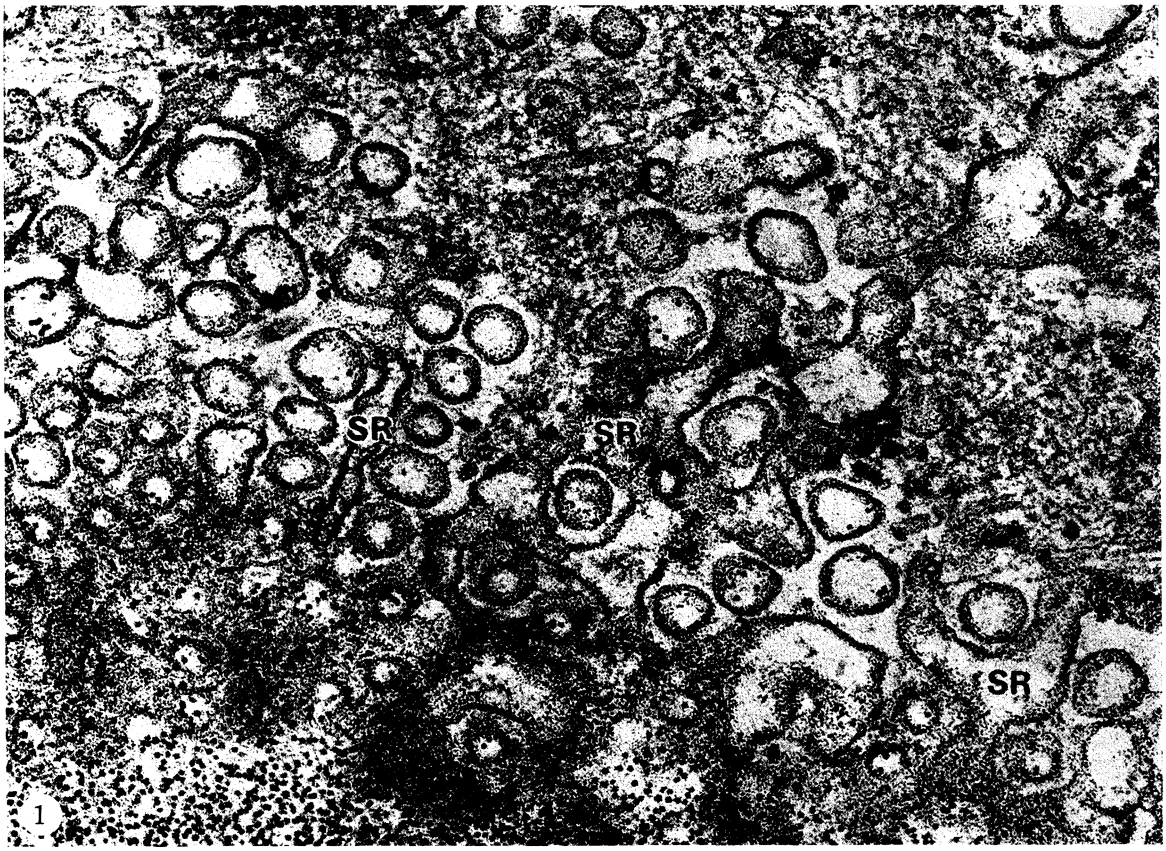

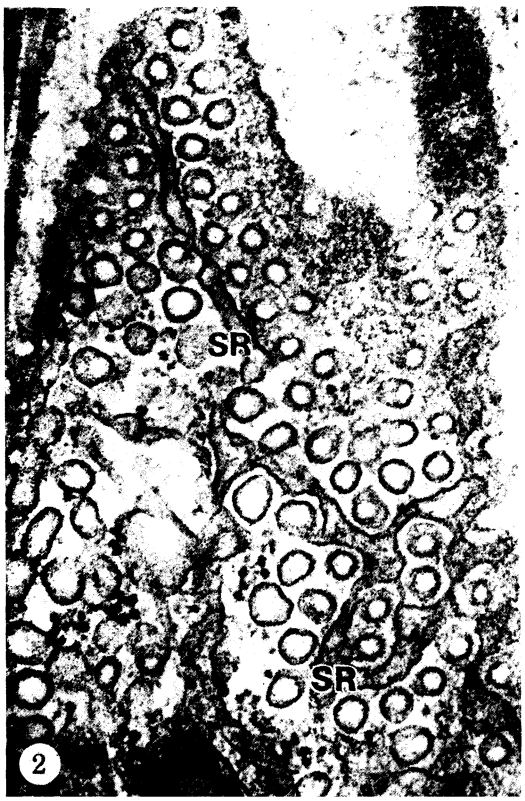

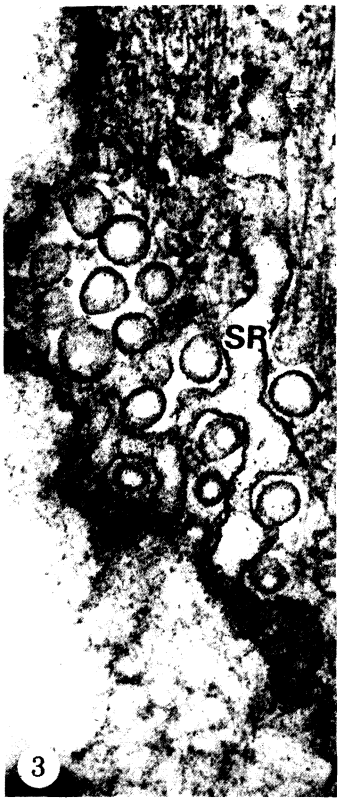

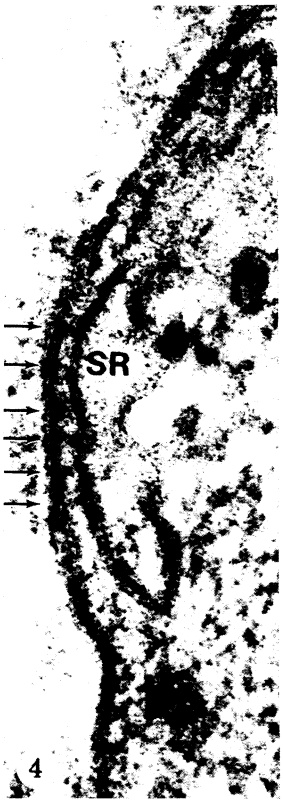

Abbreviations: SR, Sarcoplasmic reticulum; DB, dense body; PAMV, portal-anterior mesenteric vein.

FIGURE 1. Tangential section through the periphery of a rabbit PAMV smooth muscle cell showing ferritin in the extracellular space, including the surface vesicles, but not in the lumen of the SR that forms a fenestrated network around the vesicles. (Magn. × 93000.) From Devine *et al.* (1972).

FIGURE 2. Tangential section through smooth muscle in rabbit PAMV. Longitudinal tubular and fenestrated SR is coursing among the surface vesicles. (Magn. × 62000.)

FIGURE 3. Tangential section of turtle oviduct smooth muscle. A tubule of SR expands into loops around surface vesicles. (Magn. × 62000.) From Somlyo *et al.* 1971).

FIGURE 4. SR–surface membrane relationship (coupling) in rabbit PAMV smooth muscle. The approximately 10 nm gap is traversed by electron opaque material (arrows) suggestive of a periodicity of approximately 20 nm (Magn. × 207000.) From Devine *et al.* (1972).

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fashion suggested by the values cited above, because the oestrogen dominated rat uterus, a spike generating spontaneously active smooth muscle (Marshall 1962), contains a rather large volume of sarcoplasmic reticulum (Ross & Klebanoff 1967; A. P. Somlyo, unpublished observations). It is more likely that the extensive sarcoplasmic reticulum of the smooth muscle of large elastic arteries and of the oestrogen dominated uterus is related to the morphogenetic function of these smooth muscles that also elaborate the surrounding connective tissue elements (for review see Somlyo & Somlyo 1968*b*; Ross 1971; Ross & Klebanoff 1971). It may be that a large volume of sarcoplasmic reticulum, whether involved primarily in protein synthesis or in glycogen metabolism (Somlyo *et al.* 1971) incidentally also contributes to an enlarged intracellular calcium pool.

The total calcium-binding capacity of the rough sarcoplasmic reticulum in smooth muscle, however, is uncertain. The smooth and the rough sarcoplasmic reticulum are in direct communication, and deposits of strontium have been seen in the portion of smooth reticulum immediately continuous with the rough reticulum (Somlyo & Somlyo 1971*a*, *b*, and unpublished observations). Thus far, we have not been able to find, in a limited number of preliminary observations, strontium deposits directly within the lumen of a tubule covered with ribosomes. It is possible that while the concentrations of *free* calcium within the lumen of smooth and rough sarcoplasmic reticulum (subject to a diffusion gradient) are nearly identical, the total (including bound) cation may be greater in the smooth sarcoplasmic reticulum. If this were the case, then estimates of sarcoplasmic reticulum that include both the smooth and rough component would give an excessive value for calcium-binding capacity of the muscle fibre.

Peripheral elements of the sarcoplasmic reticulum approach the surface membrane, separated from it by a gap (10 to 12 nm) that is traversed by electron opaque connexions (figure 4 plate 9). These sarcoplasmic reticulum – surface membrane relations resemble the couplings of cardiac muscles (Sommer & Johnson 1968; Fawcett & McNutt 1969), and are present in both the reptilian and mammalian smooth muscles. The junctional sarcoplasmic reticulum forming close appositions with the surface membrane in smooth muscle appears to be one of the most probable sites of stored calcium that can be released by the action potential; the presence of these structures supports the mechanism of electromechanical coupling originally suggested by this laboratory (Somlyo & Somlyo 1968*a*). It is unlikely that sufficient calcium moves as a charge in the action potential to activate smooth muscle contraction (Somlyo *et al.* 1971). The hypothesis (Goodford 1967) that the twitch contractions are directly activated by the calcium current of spikes also seems unlikely in view of observations that the contractions persist for some time in calcium-free solutions (Bülbring & Tomita 1970; Somlyo *et al.* 1971).

The calcium-accumulating activity of the sarcoplasmic reticulum in vertebrate smooth muscles is suggested by the localization of strontium in smooth muscles incubated in strontium-containing solutions before fixation (Somlyo & Somlyo 1971*a*). Strontium has a sufficiently high atomic number to be electron opaque when present in the concentrations reached following such incubations and it is seen to be localized to the sarcoplasmic reticulum (and mitochondria) of smooth muscle (figures 7 to 9, plate 11). Because strontium, like calcium, can activate actomyosin ATPase and is also accumulated by isolated sarcoplasmic reticulum and mitochondrial preparations with kinetics similar to those of calcium accumulation (for references see Somlyo & Somlyo 1971*a*), these observations are highly suggestive concerning the function of the sarcoplasmic reticulum of smooth muscle as a calcium storage site. Microsomal preparations with calcium-binding activity have also been isolated from a variety of smooth

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muscles (Carsten 1969; Batra & Daniel 1971; Baudouin, Meyer, Fermandjian & Morgat 1972). Barium is not accumulated by the sarcoplasmic reticulum in smooth muscle (Somlyo & Somlyo 1971*a*).

Mitochondria often make close contacts (average 4 to 5 nm) with surface vesicles and they can accumulate electron opaque deposits of barium and strontium (Somlyo & Somlyo 1971*a*; Somlyo, Devine & Somlyo 1972). Barium accumulation in mitochondria is inhibited by oligomycin and cyanide (Somlyo & Somlyo, unpublished observation). The possibility has been considered that the mitochondrial-surface vesicle relationships are sites of cation uptake and may play an auxiliary role in regulating intracellular calcium levels (Somlyo & Somlyo 1970, 1971*a*; Somlyo *et al.* 1972; Goodford & Wolowyk 1972).

The ultrastructural findings and the residual contraction of smooth muscle in virtually calcium-free media should not be interpreted to indicate that extracellular calcium does not contribute to activation of drug-induced contractions of smooth muscle. Both radioisotope flux studies of cholinergically excited smooth muscle (Durbin & Jenkinson 1961) and the depolarization of α-adrenergically stimulated vascular smooth muscle in Na-free solutions with calcium as the external cation (Somlyo & Somlyo 1971*b*) indicate that excitatory drugs increase the permeability of smooth muscle membrane to calcium. More recently, the study of radio-calcium-movements with the lanthanum technique also verified the norepinephrine induced calcium influx into vascular smooth muscle (Van Breemen *et al.* 1973, this volume). The relative contributions of, respectively, extracellular and intracellular calcium stores to activation may vary under different experimental conditions and in different types of smooth muscle.

The surface vesicles, although possibly playing a specialized role in ion transfer between sarcoplasmic reticulum and mitochondria on the one hand, and the extracellular space on the other, are in direct communication with the extracellular space and cannot be considered a true intracellular store of calcium. Ferritin, lanthanum (Somlyo *et al.* 1971; Devine *et al.* 1972), and horseradish peroxidase (Devine & Somlyo, unpublished results) penetrate even what appear to be 'free floating' vesicles, and it is probable that the apparent lack of direct communication of such vesicles with the extracellular space in conventional electron micrographs is due to the neck being outside the plane of section or oriented in a manner tangential to the incident electron beam (Devine *et al.* 1972). There is no evidence that the vesicles observed in routine electron micrographs have a true pinocytotic function. Some surface vesicles in the smooth muscle of the turtle have a substructure consisting of approximately 10 nm striations (Somlyo *et al.* 1971): the function of these striations is unknown.

The manner in which drugs can release calcium from an intracellular store that is not in direct contact with the plasma membrane, whether a centrally located element of the sarcoplasmic reticulum or a mitochondrion, is one of the more fundamental questions of smooth muscle physiology that remains to be answered. The possibility that excitatory drugs cross the plasma membrane and act directly on the membrane system of the intracellular calcium storing organelles cannot be excluded, but seems less likely in view of the ability of large molecular mass compounds (angiotensin) to produce contractions of depolarized smooth muscle in calcium-free solutions (Somlyo & Somlyo, cited in Devine *et al.* 1972) and the recent finding (Devynck, Baudouin, Pernollet & Meyer 1972) that angiotensin receptors are located on the plasma membrane, rather than on the sarcoplasmic reticulum fraction of fresh microsomal preparations obtained from smooth muscle. It has been suggested, that a permeability change induced by drugs at the junctional sarcoplasmic reticulum forming close appositions with the plasma

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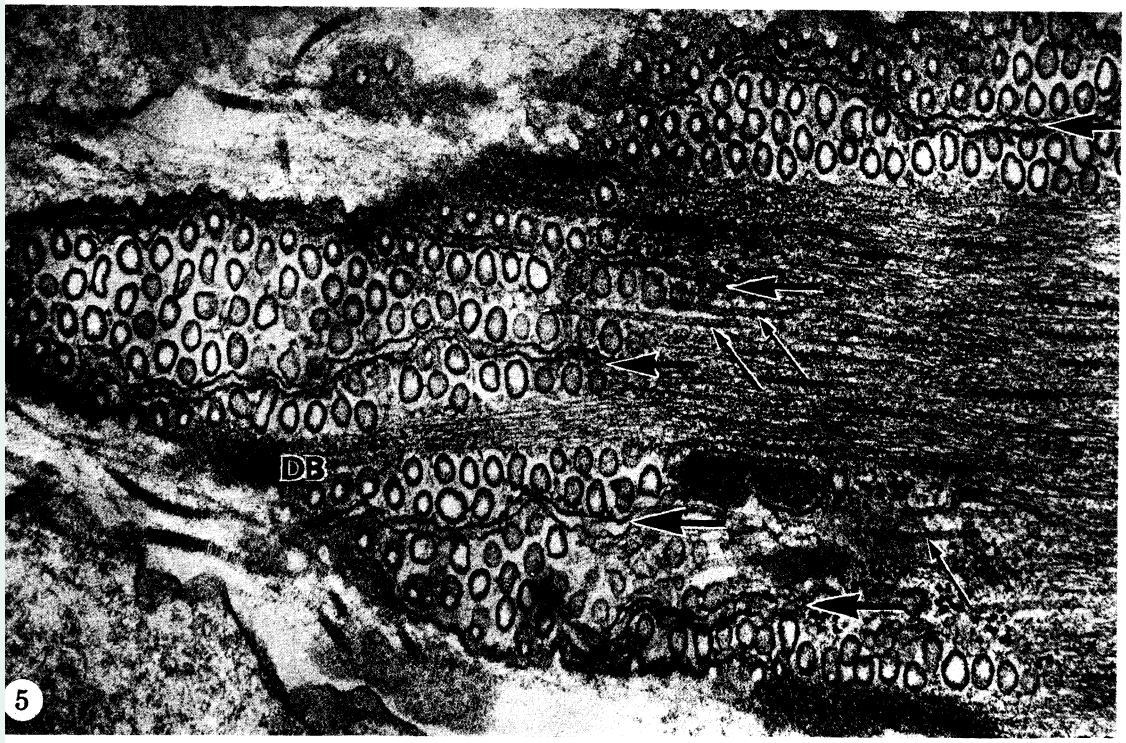

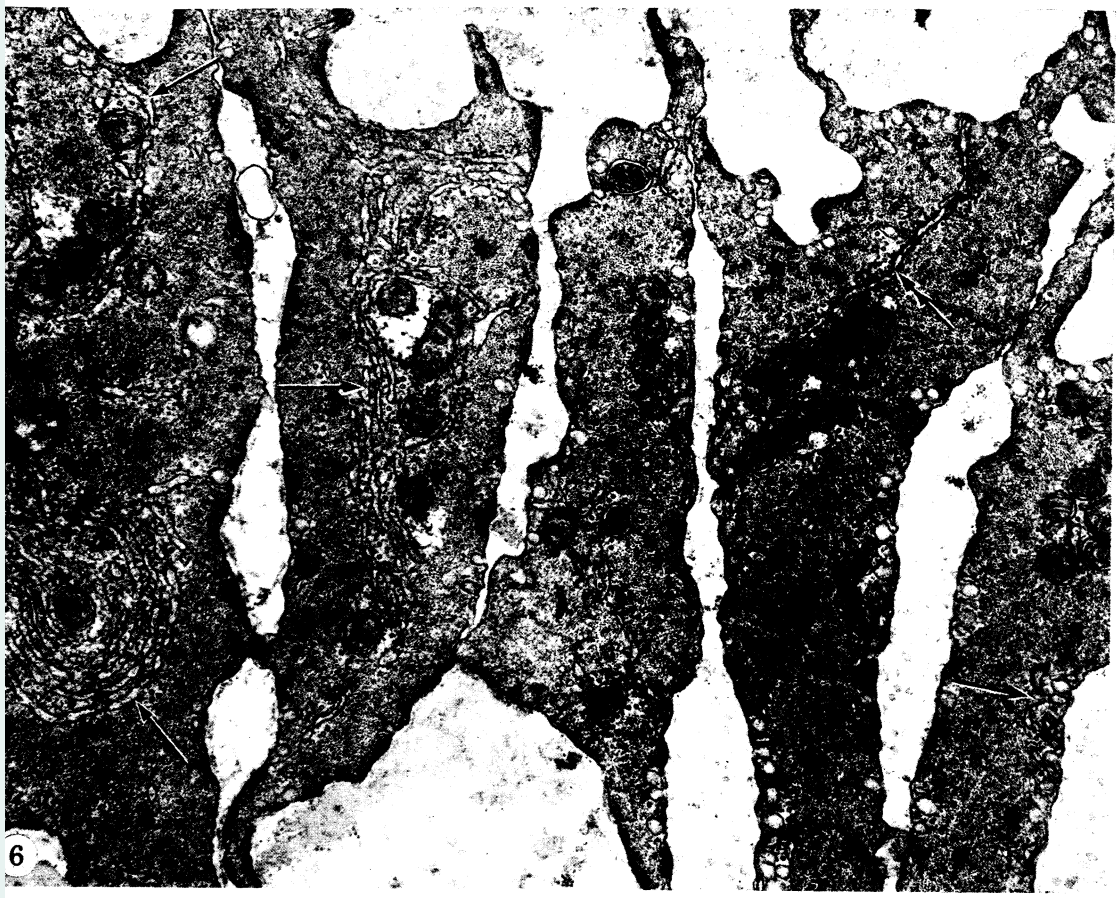

URE 5. A tangential section of rabbit PAMV showing finger-like elements of SR (arrows) running longitudinally through groups of surface vesicles separated from each other by DB. Some microtubules are also present near the surface vesicles (small arrows). (Magn. × 53000.) From Devine *et al.* (1972).

URE 6. Low magnification view of transverse section of rabbit main pulmonary artery. Note the relatively large amount of central and peripheral SR (single arrows), and the extensions of the central tubules towards the plasma membrane. Rough sarcoplasmic reticulum (double arrow). (Magn. × 25000.)

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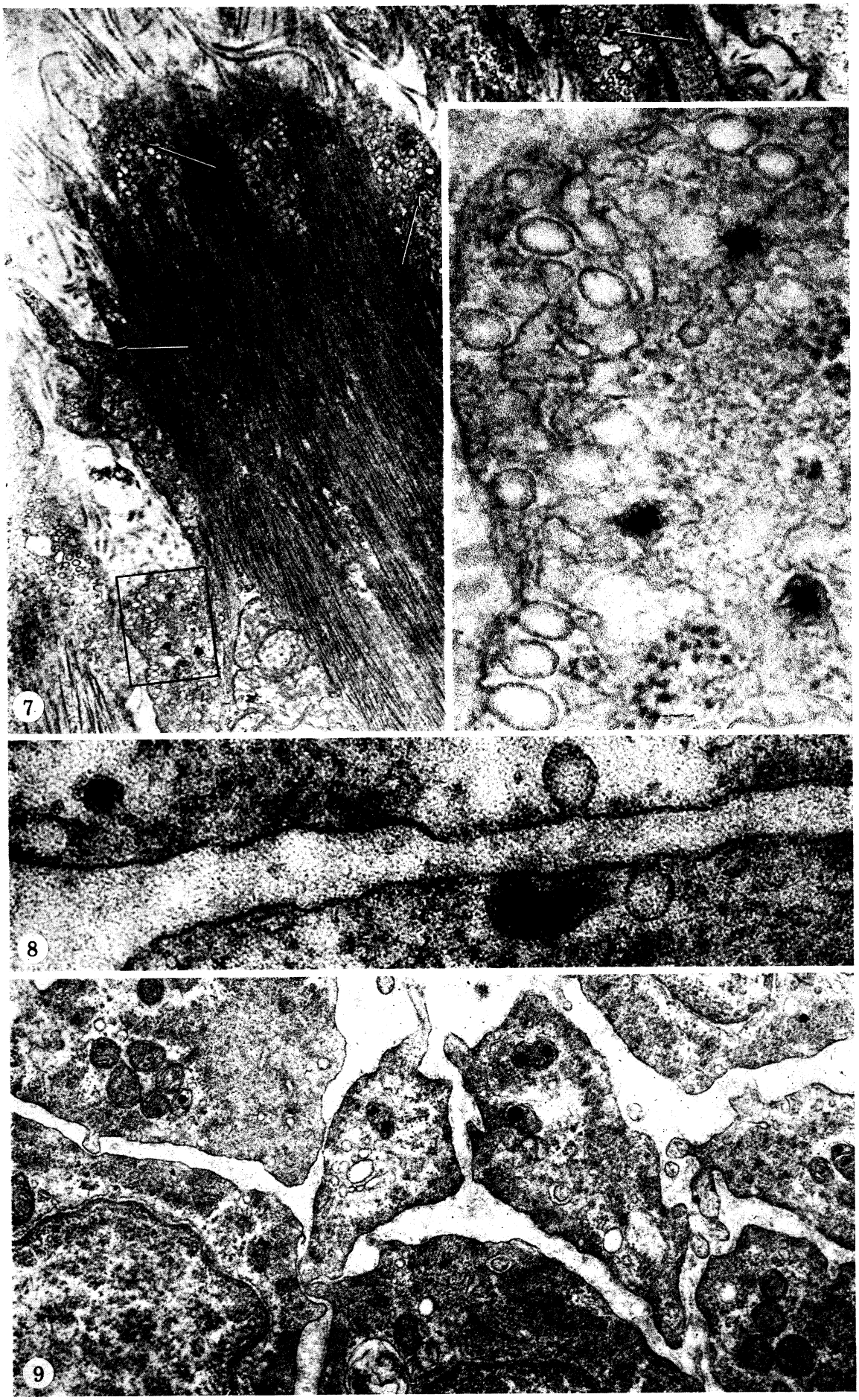

FIGURES 7 TO 9. For legend see facing page.

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membrane may be propagated as a local circuit calcium current along the surface of sarcoplasmic reticulum tubules, thus releasing the calcium from the more centrally located binding sites (Somlyo *et al.* 1972). This, and other suggested mechanisms of 'pharmaco-mechanical coupling', remain to be tested by more direct experimentation.

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Description of plate 11

Figure 7. Longitudinal-oblique section of smooth muscle in a rabbit portal-anterior mesenteric vein that had been strontium-loaded through incubation in a Na-free (sucrose substituted) solution including 10 mmol/l strontium for 1 h, followed by primary fixation in osmium. Electron opaque deposits of strontium (arrows) are in portions of the SR coursing among the surface vesicles. Thick section, block-stained with uranyl acetate. (Magn. × 25000; insert × 103000.) Strontium is also accumulated (not shown in this micrograph) in central portions of the SR and in the perinuclear space (Somlyo & Somlyo 1971 *a*, and unpublished observations).

Figure 8. Unstained transverse section of smooth muscle in rabbit PAMV incubated in Sr–Krebs solution before fixation with osmium. Electron opaque deposit of strontium is in the junctional SR. (Magn. × 120000.)

Figure 9. Transverse section of an unstained, oestrogen-treated rat uterus, loaded with strontium, primary fixation with osmium. Large intramitochondrial deposits of strontium are present. (Magn. × 25000.)

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Discussion

J. S. Gillespie: If pulmonary arterial muscle is made to contract in a calcium free solution can you show displacement of barium or strontium from the sarcoplasmic reticulum or mitochondria?

A. P. Somlyo: We have not yet shown the release of barium or strontium during contraction and, since the question is a highly pertinent one, we hope to do these experiments within the near future. We suspect that barium does not directly activate the contractile proteins, but that it produces contractions by increasing the free cytoplasmic concentration of calcium. Strontium, judging from the contractions of Ca-depleted rabbit portal-anterior mesenteric veins in the Sr-containing solutions, probably activates the troponin–tropomyosin–actomyosin system.

Dr P. Meyer (*Paris*): In a previous study we have demonstrated in rabbit aortae the existence of microsomal membranes capable of binding a fairly large amount of calcium in the presence of Mg ATP. Angiotensin II was found to increase the release of calcium from the microsomal membranes (Baudouin, M., Meyer P., Fermandjian S. & Morgat J. L. 1972 *Nature, Lond.* **235**, 336). With the use of ultracentrifugation in Ficoll gradients, and different marker enzymes (5′ nucleotidase, adenyl cyclase) we were recently able to separate the plasma membranes from the intracellular membranes. Specific receptors of [^{3}H]-angiotensin II were localized in plasma membranes. In the absence of Mg ATP, the membraneous uptake of calcium was low and occurred equally in plasma and intracellular membranes. The uptake of calcium was increased by Mg ATP in the plasma membranes only. It seems therefore that different membraneous systems capable of binding calcium exist in smooth muscle cells, and that the mechanisms of calcium binding are not similar in the different calcium stores.

M. W. Wolowyk: Both you and Dr Gabella have suggested that the reticulum and mitochondria observed in smooth muscle are primarily involved with the provision of calcium for the contractile response, but your studies only show uptake of electron-dense cations by

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these structures and not release from them. However, under normal physiological conditions there is sufficient exchangeable extracellular and membrane-bound calcium to initiate and maintain contraction (Goodford, J., *J. Physiol. Lond.* **192**, 145–157 (1967)), and it seems probable that the reticulum and mitochondria might function to extrude calcium and other cations from the cell. Why have you not considered this possibility?

A. P. Somlyo: The ultrastructural findings reported here are not intended to suggest that the intracellular storage sites are the only sources of activator calcium under physiological conditions and that extracellular calcium makes no contribution to the activation of contraction in smooth muscle. In fact, we have presented evidence in the past showing that norepinephrine does increase the permeability of the surface membrane to calcium (p. 219, *Vascular neuroeffector systems* (Bevan, Furchgott, Maxwell & Somlyo, editors), Basel: Karger, 1971). Nevertheless, the amount of calcium that could be carried as a charge in the action potential does not appear to raise intracellular (bound and free) calcium concentrations sufficiently to activate a twitch (*J. Cell Biol.* **51**, 722–741 (1971); **52**, 690–718 (1972)) and spontaneous twitch contractions persist in calcium-free solutions (see references). By analogy with cardiac muscle, we suggest that the junctional sarcoplasmic reticulum in close apposition to the surface membrane is the calcium accumulating site from which the action potentials release calcium in initiating the twitch contraction.

Some calcium bound to the surface membrane may function as trigger calcium or, as suggested in many systems, perhaps regulates the permeability of the membrane. The amount of membrane-bound calcium that Dr Wolowyk refers to is presumably estimated from uranyl acetate binding to glutaraldehyde-fixed (Goodford & Wolowyk, *J. Physiol., Lond.* 1972) smooth muscle membranes: it is yet to be shown that the cation binding capacity of the unfixed membranes is similar. Calcium binding by unfixed red cell membranes, in the presence of physiological ATP concentrations, is slight (Chau-Wong & Seeman, *Biochim. biophys. Acta* **241**, 473–782 (1971)).

Finally, there is no evidence yet that mitochondria can release calcium to activate contraction and even the view shared by Dr Wolowyk and ourselves, that mitochondria may contribute to relaxation by the uptake of calcium, is speculative. It remains to be shown that mitochondrial calcium uptake occurs at the low concentrations required to relax smooth muscle, although the possibility is suggested by some of our electron micrographs showing strontium accumulation by mitochondria in smooth muscles that were spontaneously contracting (in a Ca-free, Sr-containing solution) before fixation. We are in agreement that there may be multiple sources of activator calcium and that mitochondria and the sarcoplasmic reticulum may participate in the uptake and extrusion of calcium from smooth muscle.

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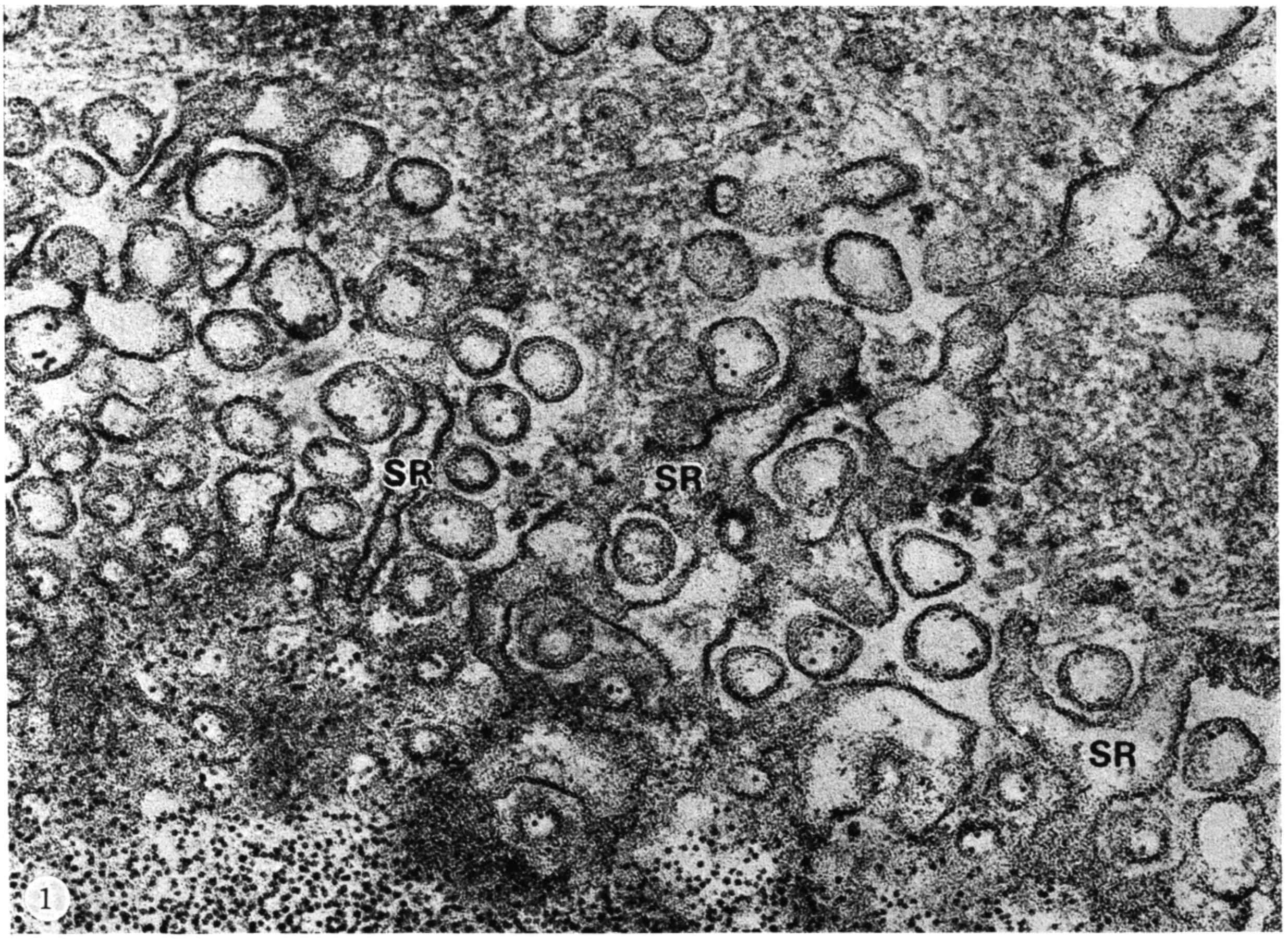

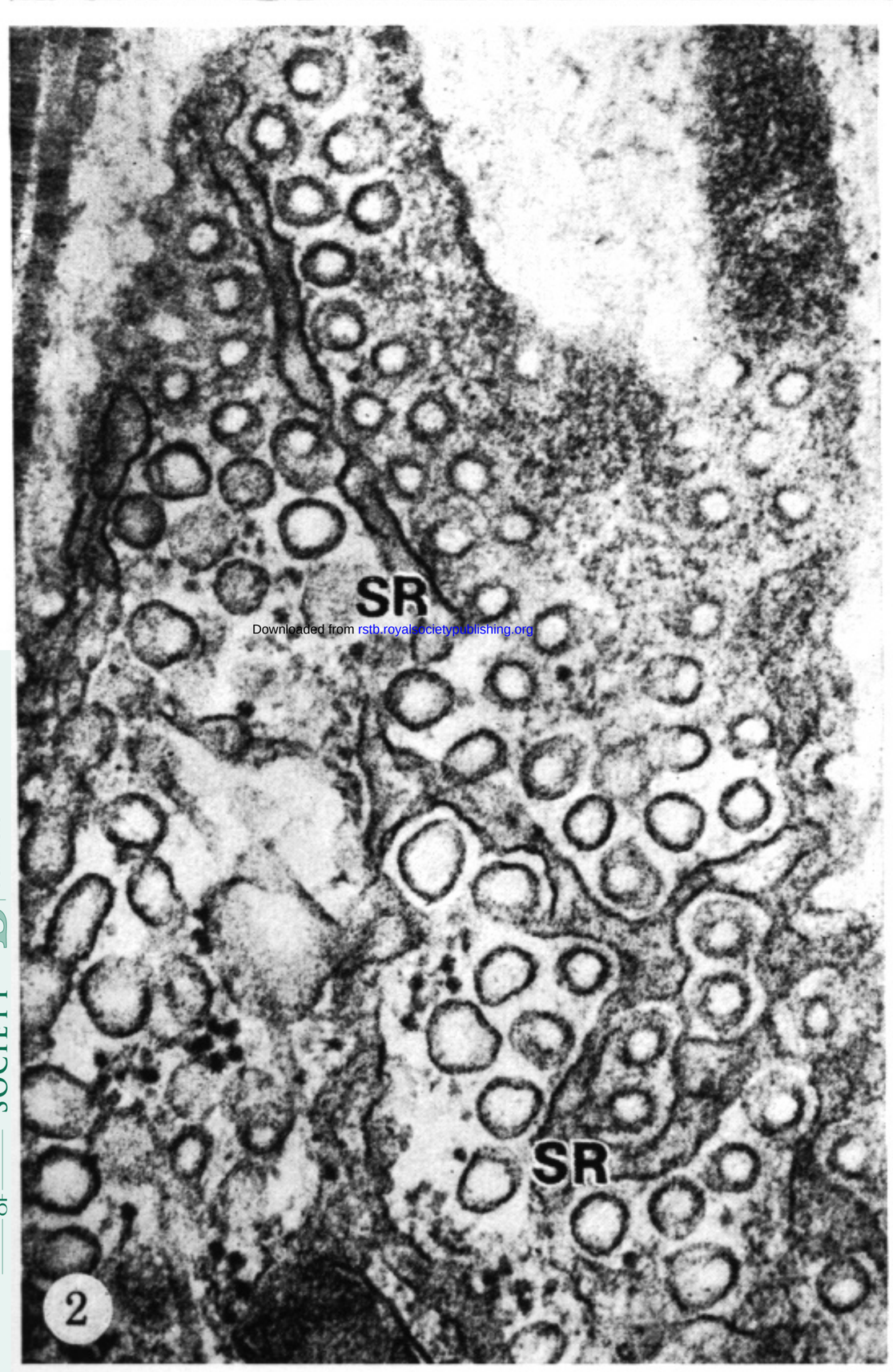

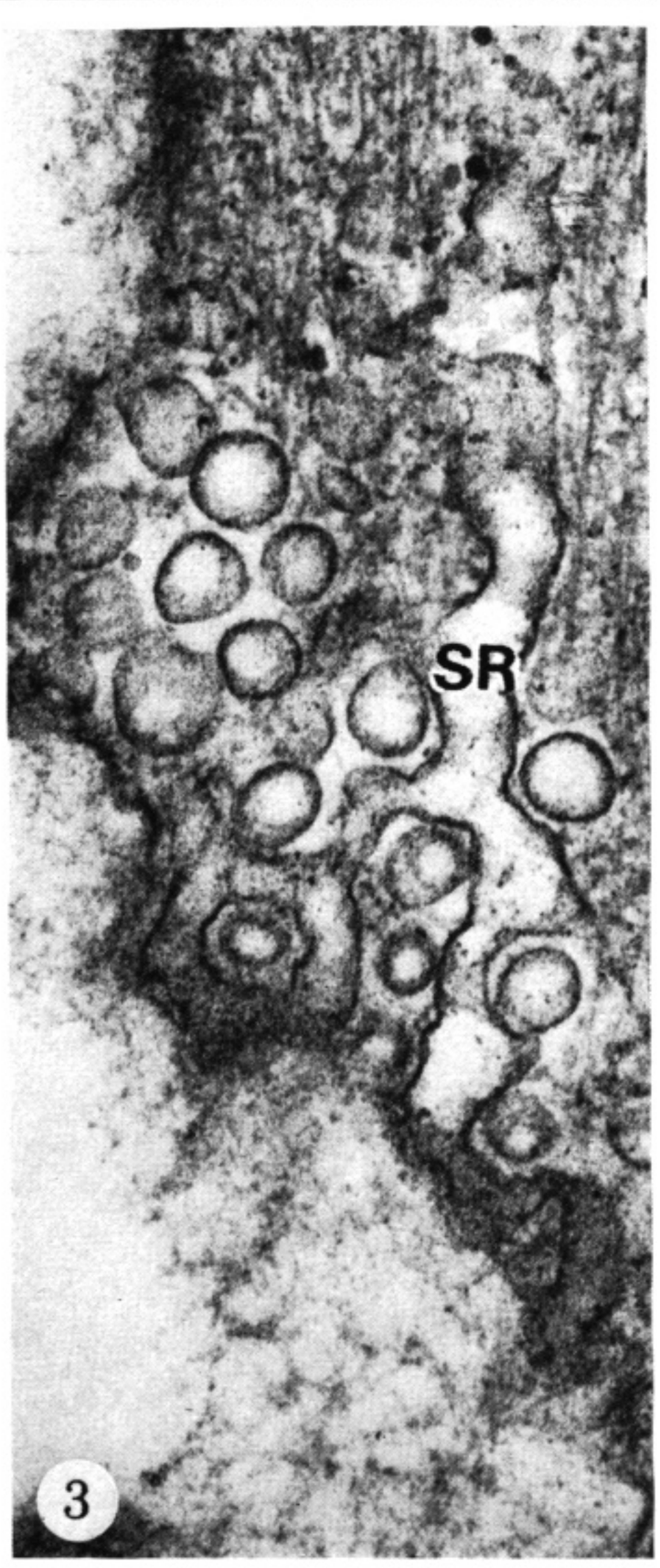

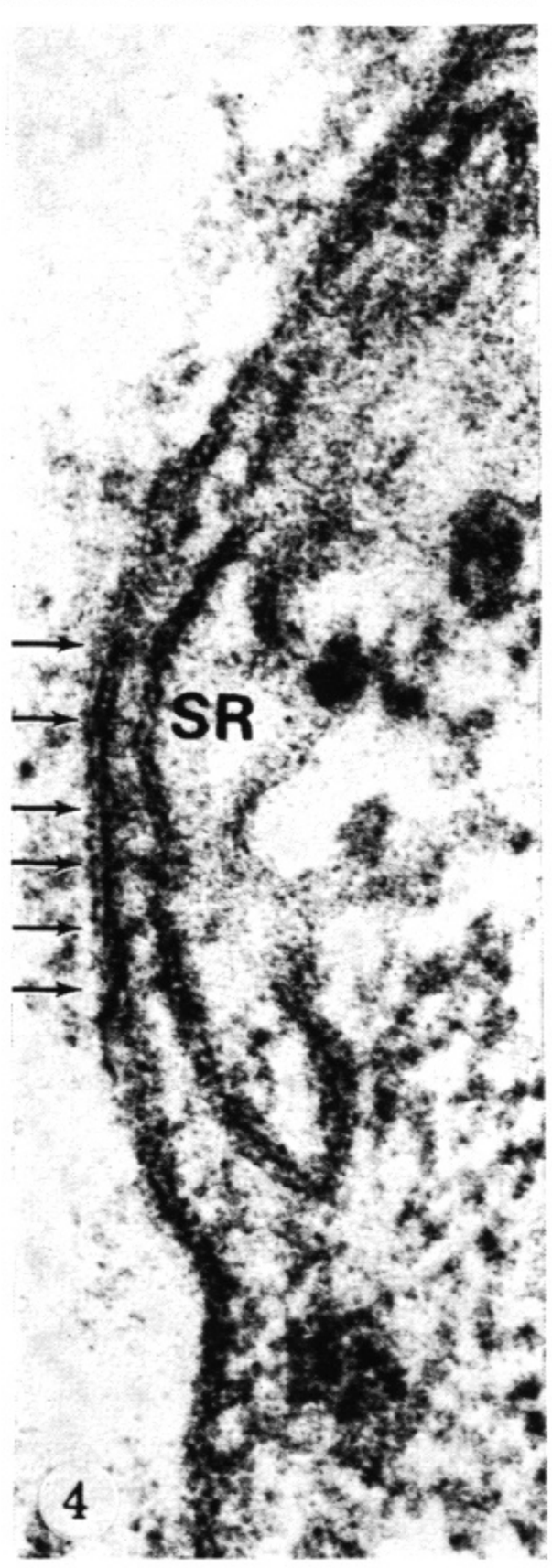

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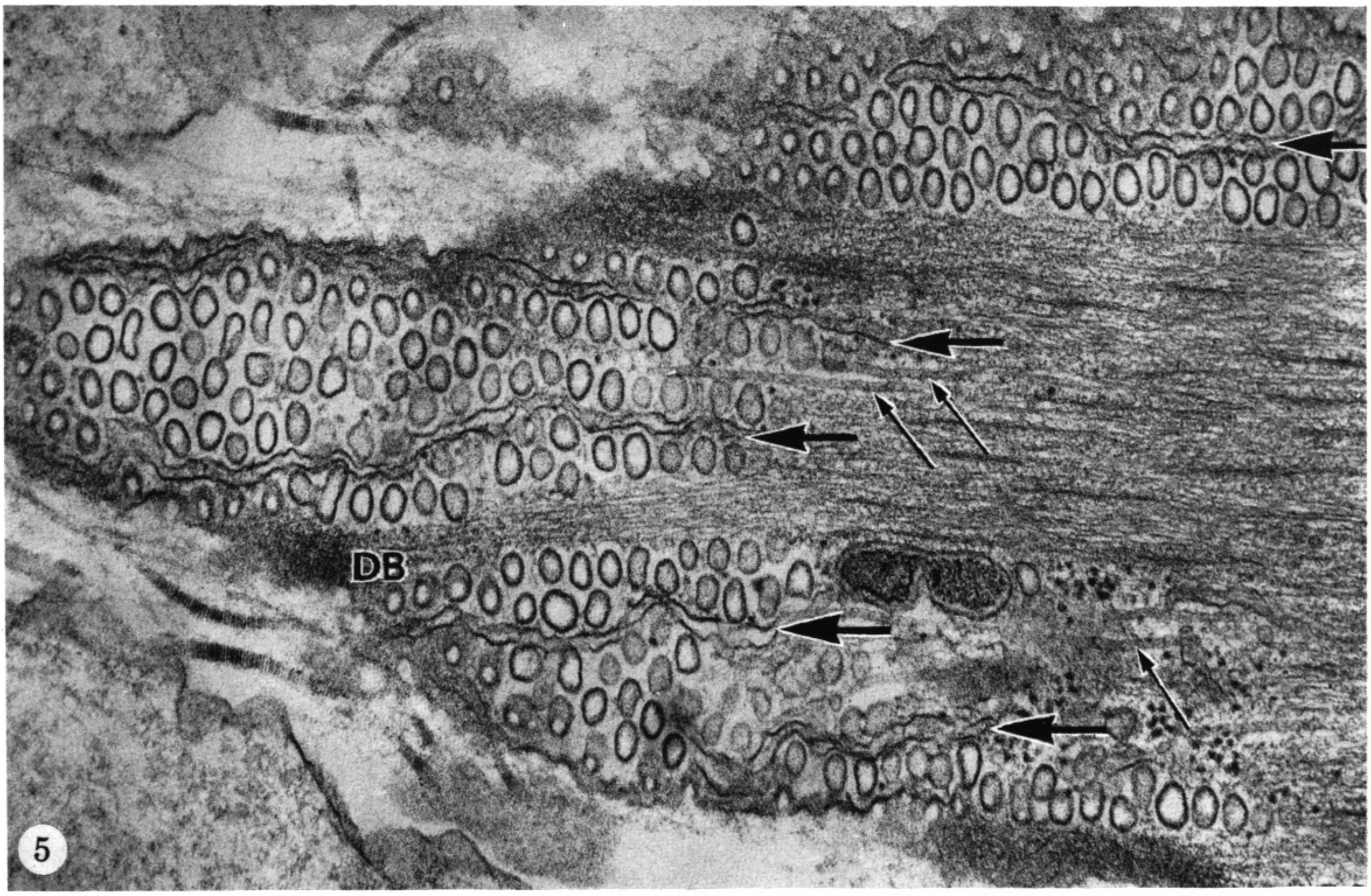
Abbreviations: SR, Sarcoplasmic reticulum; DB, dense body; PAMV, portal-anterior mesenteric vein.

FIGURE 1. Tangential section through the periphery of a rabbit PAMV smooth muscle cell showing ferritin in the extracellular space, including the surface vesicles, but not in the lumen of the SR that forms a fenestrated network around the vesicles. (Magn. ×93000.) From Devine *et al.* (1972).

FIGURE 2. Tangential section through smooth muscle in rabbit PAMV. Longitudinal tubular and fenestrated SR is coursing among the surface vesicles. (Magn. ×62000.)

FIGURE 3. Tangential section of turtle oviduct smooth muscle. A tubule of SR expands into loops around surface vesicles. (Magn. ×62000.) From Somlyo *et al.* 1971).

FIGURE 4. SR–surface membrane relationship (coupling) in rabbit PAMV smooth muscle. The approximately 10 nm gap is traversed by electron opaque material (arrows) suggestive of a periodicity of approximately 20 nm (Magn. ×207000.) From Devine *et al.* (1972).



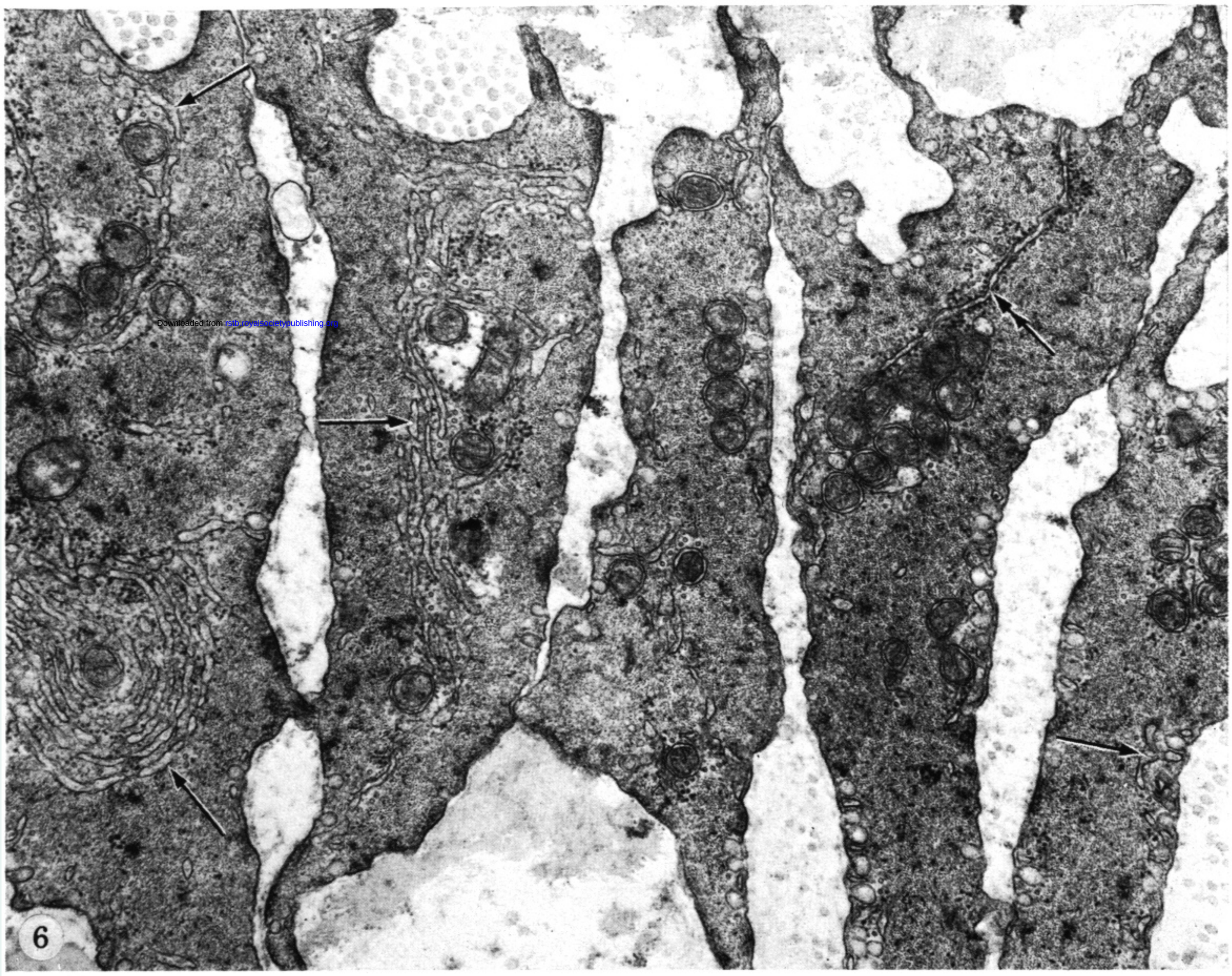


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FIGURE 5. A tangential section of rabbit PAMV showing finger-like elements of SR (arrows) running longitudinally through groups of surface vesicles separated from each other by DB. Some microtubules are also present near the surface vesicles (small arrows). (Magn. ×53000.) From Devine *et al.* (1972).

FIGURE 6. Low magnification view of transverse section of rabbit main pulmonary artery. Note the relatively large amount of central and peripheral SR (single arrows), and the extensions of the central tubules towards the plasma membrane. Rough sarcoplasmic reticulum (double arrow). (Magn. ×25000.)

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FIGURES 7 TO 9. For legend see facing page.