

FREEZE-FRACTURE STUDIES OF NEXUSES BETWEEN SMOOTH MUSCLE CELLS

Close Relationship to Sarcoplasmic Reticulum

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ABSTRACT

The freeze-fracture appearance of the nexus was compared in the smooth muscle of guinea pig sphincter pupillae, portal vein, pulmonary artery, taenia coli, uretzr, and vas deferens, mouse vas deferens, chicken gizzard and anterior mesenteric artery, and toad stomach. Nexuses are particularly numerous in the guinea pig sphincter pupillae; they are usually oval and their average area is $0.15 \mu\text{m}^2$, although some as large as $0.6 \mu\text{m}^2$ were seen. Small aggregations of particles were observed which would not be recognizable as nexuses in thin section. What constitutes the minimum size of a nexus is discussed. It is estimated that the number of nexuses per cell in this preparation is of the order of tens rather than hundreds. All nexuses examined had 6–9-nm particles in the PF face, with corresponding 3–4-nm pits on the EF face forming a polygonal tending towards a hexagonal lattice. The nexuses are arranged in rows parallel to the main axis of the cell, usually alternating with longitudinal rows of plasmalemmal vesicles. Many nexuses in the guinea pig sphincter pupillae, chicken gizzard, and toad stomach show a close relationship with sarcoplasmic reticulum. The possibility that this may have some role in current flow across this specialized junction is discussed.

Areas of close apposition or "bridges" between apposing membranes of smooth muscle cells were first recognized by Bergman (6), and by Prosser et al. (30); this type of junction was termed the "nexus" by Dewey and Barr (16, 17). Nexuses have since been recognized in a variety of smooth muscle preparations, including chicken and pigeon gizzard, guinea pig taenia coli, mouse and guinea pig vas deferens (2, 13), rabbit pulmonary artery (15), dog duodenum (22), rat duodenum (18),

and guinea pig ileum and sphincter pupillae (19, 20). They have also been described in cultured smooth muscle (11).

The nexuses have been considered by most workers to constitute the morphological basis of the low resistance pathways that allow electrotonic coupling of activity between adjacent smooth muscle cells within muscle effector bundles (4, 16). However, doubts have been raised about this conclusion since the observation that very few nexuses

are present in the longitudinal muscle of the intestine of dog (22) and guinea pig (19): both of these preparations exhibit propagated action potentials. Further, nexuses have been described in rat and mouse vas deferens, where every cell appears to be innervated (9).

High resolution transmission electron microscopy has revealed that most, if not all nexuses between smooth muscle cells consist of "gap junctions" (32, 35). The freeze-fracture technique has greatly contributed to studies of the detailed ultrastructure of intercellular junctions (27). Thus, in the present study an attempt is made to clarify the nature and role of smooth muscle nexuses, by comparing their freeze-fracture appearance in a variety of preparations where electrical coupling and innervation density have been studied. The preparations selected were chicken gizzard and anterior mesenteric artery, mouse vas deferens, guinea pig taenia coli, ureter, vas deferens, sphincter pupillae, portal vein, and pulmonary artery, and toad stomach. Two previous papers have described the freeze-fracture appearance of nexuses in smooth muscle, but these were confined to preparations of the guinea pig taenia coli (21, 37).

MATERIALS AND METHODS

The following smooth muscles were investigated: chicken gizzard and anterior mesenteric artery; stomach of toad (*Bufo marinus*); guinea pig pulmonary artery, portal vein, taenia coli, ureter, vas deferens, and sphincter pupillae; and mouse vas deferens.

Tissues were fixed in 0.1 M cacodylate-buffered 2.5% glutaraldehyde (pH 7.3), infiltrated with 25–30% glycerol in cacodylate buffer for 60 min before being placed on gold disks and frozen in liquid Freon 22 and then transferred to liquid nitrogen. Nexuses were not found in all tissues fixed in this way, and with some tissues other pretreatment and fixation methods were therefore investigated. The taenia coli and portal vein were tied to wooden sticks and kept at their approximate in vivo length for 15–30 min before fixation, in some instances, since better preservation of myofilaments and sarcoplasmic reticulum after this treatment has been reported (15, 33). Perfusion fixation of the pulmonary artery in 1.5–2.5% glutaraldehyde in 0.1 M cacodylate buffer through the left ventricle results in excellent preservation and alignment of smooth muscle cells and connective tissue elements. The entire eye of the guinea pig was placed directly in the fixative and the iris dissected out. Fixed tissue stored in buffer and glycerol below -10°C does not differ from freshly fixed tissue (14). Intramembranous particles do not aggregate after prefixation in glutaraldehyde (25).

The frozen tissue was placed on the -150°C cold

stage of a Balzers BAF 300 freeze-etch device, (Balzers High Vacuum Corp., Santa Ana, Calif.) evacuated to 5×10^{-6} Torr, warmed to -100°C , and fractured with a cold metal blade at -150°C , shadowed at 45° with platinum-carbon, and coated from directly above with carbon to produce the replica, by using either resistance electrodes or electron beam evaporation (see, e.g., reference 28). The tissue was digested from the replica with sodium hypochlorite solution, and the replicas were viewed in a JEOL 100B, a Philips EM200, or a Philips EM300 electron microscope.

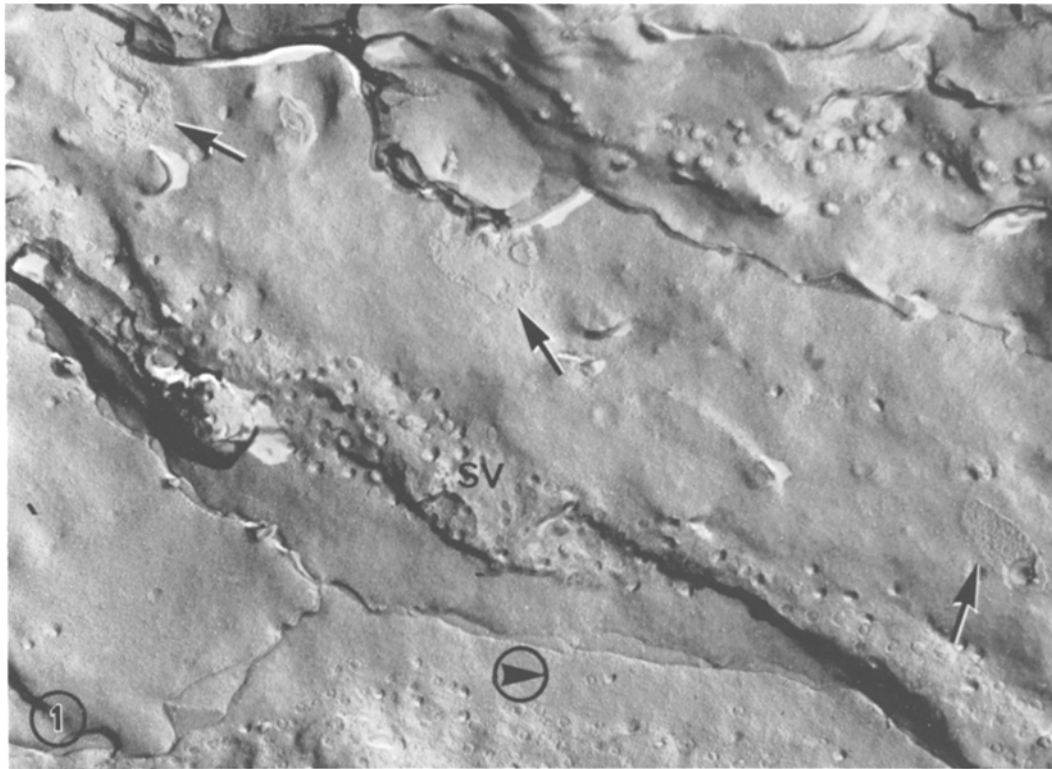
In freeze-fractured membranes, the fracture plane is through the membrane rather than either side of it (7, 8). The conventional terminology adopted for exposed fracture faces is that recently proposed by leading exponents of freeze-fracture (8). The PF face is that fracture face with the cytoplasm behind it and the EF face is that fracture face with the extracellular space behind it. Neither fracture face is the true cell surface. Natural membrane surfaces, PS and ES faces, may be revealed only by deep-etching procedures and are not considered. When the fracture plane passes through a junctional region, the exposed faces present a complex picture. The face with particles 6–8 nm in diameter with a 9–10-nm center to center spacing is the PF face, and the complementary face (12) with depression or pits 3–4 nm in diameter is the EF face.

RESULTS

Guinea Pig Sphincter Pupillae

Nexuses were very numerous in the sphincter pupillae of the guinea pig. As many as three were often observed on a single area of exposed smooth muscle cell surface (Fig. 1). In one extreme example, in one montage area where $600 \mu\text{m}^2$ of cell surface could be examined, there were 33 nexuses. They comprised $4.5 \mu\text{m}^2$ of the cell surface, i.e. 0.75% of the cell surface observed. Most of the nexuses observed were oval, and lay between the bands of plasmalemmal vesicles (also termed surface vesicles, or caveolae intracellulares). They ranged in size from $0.001 \mu\text{m}^2$ to $0.6 \mu\text{m}^2$, with a preponderance of the small nexuses (Fig. 2). In general, the larger the nexus, the greater the separation from neighboring nexuses.

All the nexuses in the sphincter pupillae (and other smooth muscle cells described below) had 6–9-nm particles on the PF face, with corresponding 3–4-nm pits on the EF face (Figs. 3–6). Exact correlation of particles and pits is difficult, since the pits are partially filled with shadowing material and the particle size is increased for the same reason. Nevertheless, the spacing of particles and pits shows a close correspondence, although some



All tissues used in freeze-fracture are from glutaraldehyde-fixed tissue infiltrated with 25% glycerol. The direction of shadowing is indicated by the circled arrowhead. *EF*, EF face of cell or sarcoplasmic reticulum membrane; *PF*, PF face of cell membrane; *SR*, sarcoplasmic reticulum; *SV*, surface vesicle.

FIGURE 1 An area of sphincter pupillae of guinea pig iris showing three nexuses (arrows) on a single smooth muscle cell. $\times 24,000$.

dislocation of the hexagonal array to form a polygonal array does occur. Usually, complete nexuses were observed, but the tissue also fractured in such a way as to reveal elements of the underlying sarcoplasmic reticulum EF face (14) (Fig. 6).

Chick Gizzard and Toad Stomach

In the chick gizzard (Figs. 7, 8) and toad stomach (Fig. 13), the nexus was relatively common. Although the sampling of nexuses in chicken gizzard was small, an average nexus size of $0.06 \mu\text{m}^2$ was observed which was calculated to contain 800 particles (hexagonal spacing of 10 nm and a particle size of 6–9 nm). In many instances in the chick gizzard, several nexuses were found in the one smooth muscle cell (Fig. 7). The pits or depressions on the EF face of the cell membrane were seen in some nexuses (Figs. 4, 6) with a spacing similar to that of the particles, although the actual

appearance and clarity depends on shadow angle (26) and other factors, such as replica quality. Elements of the sarcoplasmic reticulum membranes were also noted beneath the nexus in these tissues (Figs. 7, 8).

Guinea Pig Pulmonary Artery

Nexuses were found in pulmonary artery smooth muscle (Figs. 10, 11) but were not as common as in the chick gizzard or toad stomach. Particulate areas, considered to be nexuses, appeared either as hexagonal arrays (Fig. 11) or as small irregular groups (Fig. 10) representing a possible rudimentary nexus.

Guinea Pig Taenia coli

The taenia coli did not have large nexuses. Occasionally, there were easily recognized nexuses but usually these areas contained too few particles

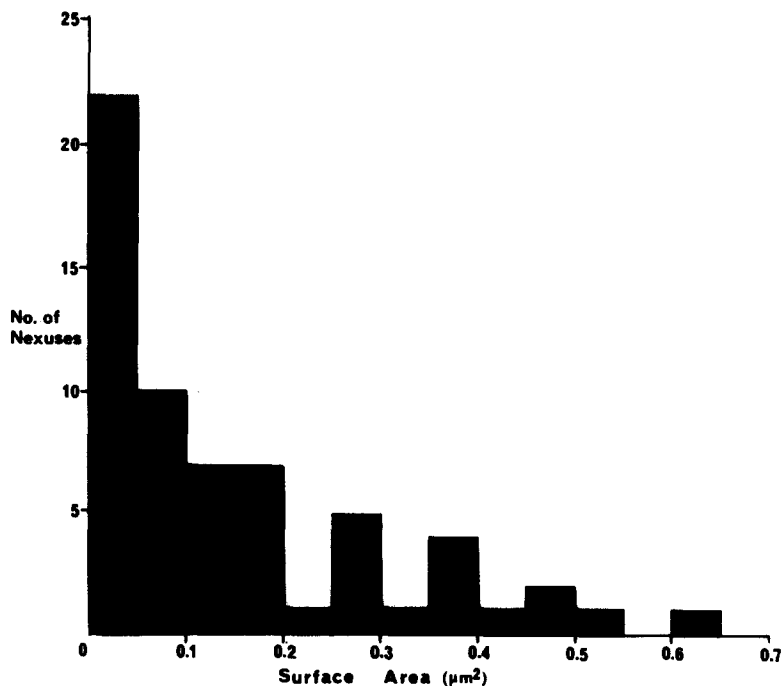


FIGURE 2 Histogram showing the surface area occupied by 62 nexuses observed in the guinea pig sphincter pupillae.

to be definitely designated as nexuses, despite some particle aggregation and close apposition of neighboring cell membranes (Fig. 9). In one instance there was a row of particles (double arrow, Fig. 9) similar to that described by Friend and Gilula (18).

Guinea Pig Portal Vein, Ureter, and Vas Deferens, Mouse Vas Deferens, and Chicken Anterior Mesenteric Artery

In muscle cells from the guinea pig portal vein and chicken anterior mesenteric artery (Fig. 12), many instances of close membrane apposition were seen and, occasionally, a few aggregated particles were noted at these regions, but there were no extensive nexuses. No well defined nexuses were found in the longitudinal muscle of guinea pig ureter or vas deferens or in longitudinal muscle of mouse vas deferens.

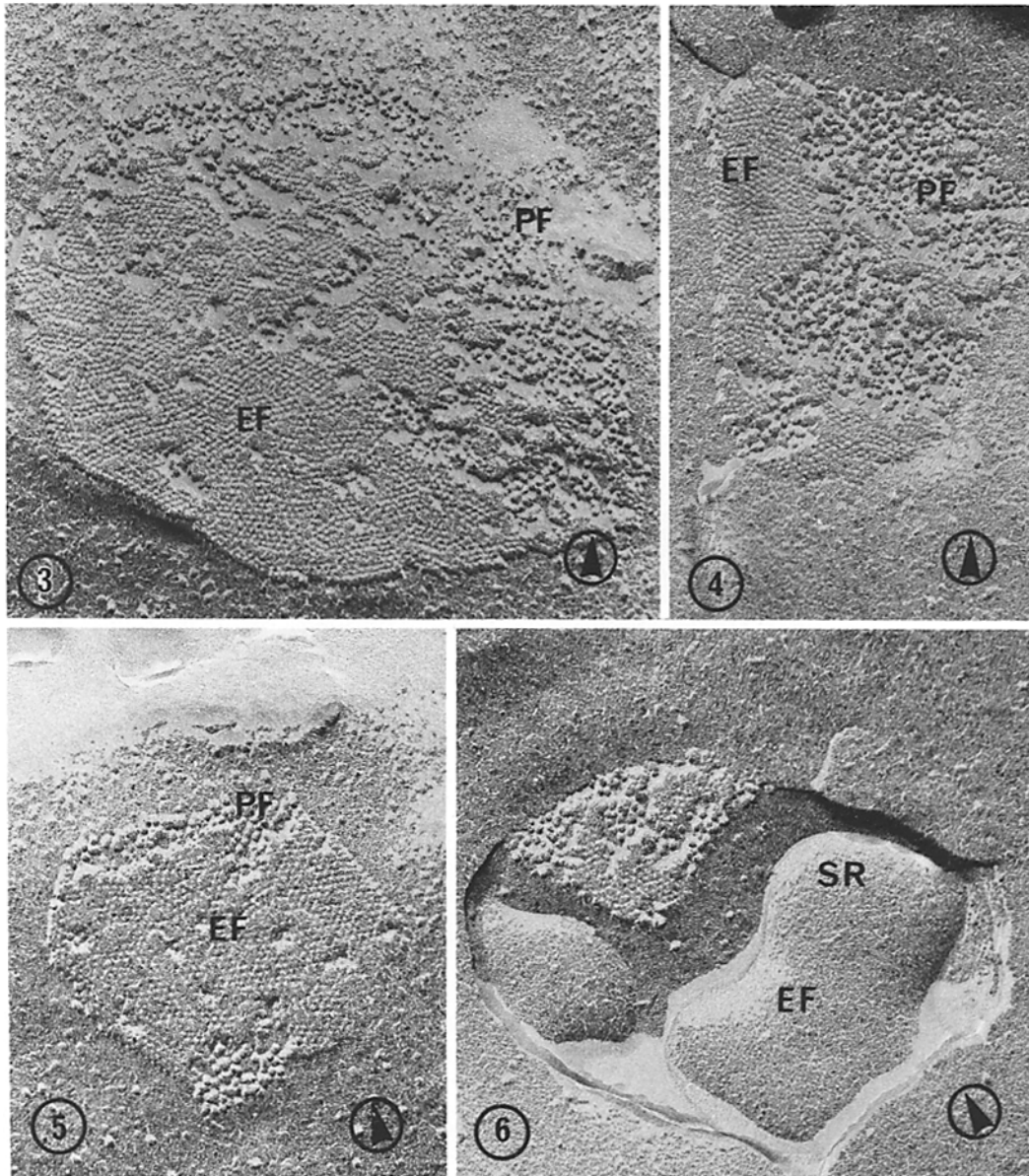
DISCUSSION

A comparison of the freeze-fracture appearance of different smooth muscles has shown that nexuses are a common feature, but there appears to be

considerable variation in the size and number of nexuses in different systems. All typical nexuses had the characteristic hexagonal particle array on the PF face, although the pits on the EF face could not always be distinguished.

The presence of nexuses with 6-9-nm particles on the PF face and pits on the EF face was evident in muscle cells of the chicken gizzard and guinea pig sphincter pupillae, toad stomach, guinea pig pulmonary artery and taenia coli, but the scarcity of such particles in the case of the longitudinal muscle from chicken anterior mesenteric artery and guinea pig portal vein made the presence of a nexus uncertain in these cells. Often, there were aggregations of particles at regions where the two adjacent cell membranes were extremely close together, but they were not in a hexagonal array. Smooth muscle cells with typical nexuses had occasional regions of close membrane apposition, with groups of a few particles at the junction region; these appeared to be similar to the particle aggregations seen in cells with no observable nexuses of the standard form.

Measurements taken from the fractured surfaces of smooth muscle cells in the guinea pig sphincter pupillae usually revealed two to four



FIGURES 3-5 High magnification views of nexuses in the guinea pig sphincter pupillae showing 8-9-nm particles on the PF face and 3-4-nm pits on the EF face. The hexagonal arrangement of the pits on the EF face also shows some dislocations of the lattice. All figures, $\times 100,000$.

FIGURE 6 A nexus with associated sarcoplasmic reticulum (SR) revealed when the membrane has been removed. $\times 100,000$.

well defined nexuses for any area ($10-16 \mu\text{m}^2$) of cell examined. Since the surface area of a smooth muscle cell observed with this method was more than 1% of the total cell surface (estimated on the

basis of a cylindrical cell $300 \mu\text{m}$ long, $2 \mu\text{m}$ diameter), a rough estimate for the number of nexuses per cell in the preparation would be in the order of 100. The nexus in this and other prepara-

tions is usually elongated, with the major axis parallel to the longitudinal axis of the cell, alternating with rows of vesicles, confirming the conclusion of Gabella (20) from his studies of thin sections.

The nexus is seen in freeze-fracture studies to be a grouping of particles in a hexagonal lattice, and in sectioned material a similar hexagonal array of particles is outlined by lanthanum (see review by McNutt and Weinstein [27]). The nexus has been implicated as a low resistance pathway between cells (2-5, 23). The particles seen on the PF face of the nexus have been suggested by Chalcraft and Bullivant (12) to match up with the pits on the EF face. Small 1.5-2.5-nm central dots seen in nexus subunits with lanthanum treatment or freeze-fracture studies have been suggested as possibly representing the morphological site of channels which traverse the nexus (26) and could account for electrical coupling between cells.

In freeze-fracture replicas, it seems reasonable that the particles of the hexagonal array correspond to the subunits of the nexus seen in thin sections. An interesting question is whether a certain minimum number of nexus subunits or even a particular arrangement of subunits is necessary for communication pathways between cells or whether communication can be achieved by any number of channels or subunits. The particles at a nexus are possibly chemically different from the particles seen elsewhere on the membrane; but at present no distinguishing structural features can be seen. A nexus therefore is easily recognized if a hexagonal array of subunit particles is present, but may be missed if only two or three subunit particles are present. Unequivocal isolated pits corresponding to the small areas of particles would not be easily observed on the EF face and could not therefore be used in any diagnosis of a nexus. In sectioned material it is even more difficult to recognise a nexus, especially if it is small and not correctly oriented to the incident electron beam, or if, in large plaque-like close appositions of the cell membrane, occasional areas of punctate nexus are present.

Nexuses do not always occupy uniform oval areas and many different shapes occur. In photoreceptor cells in the retina there are rows of particles and, occasionally, circles of particles (31). The rows of particles in this study (Fig. 9) occasionally show some resemblance to the rows of particles in the retina. Pits on the EF face comple-

mentary to particles on the PF face were best seen in the iris in this study, although in many cases there is a change in the texture of the EF face surface, possibly reflecting some unusual fracturing properties at the junctional region.

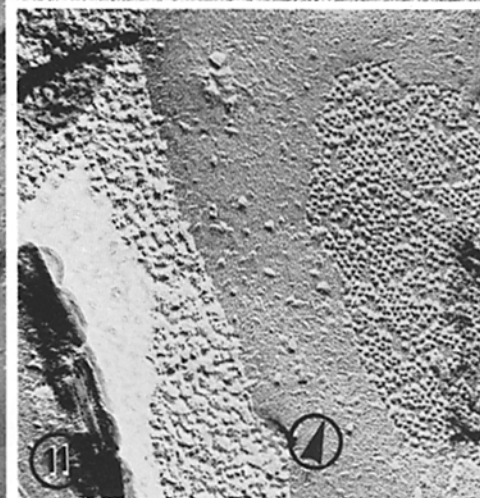
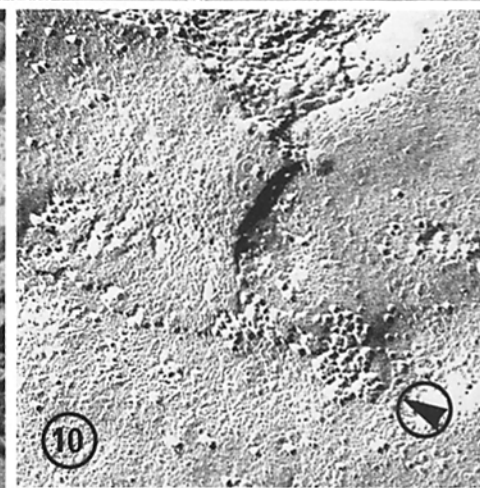
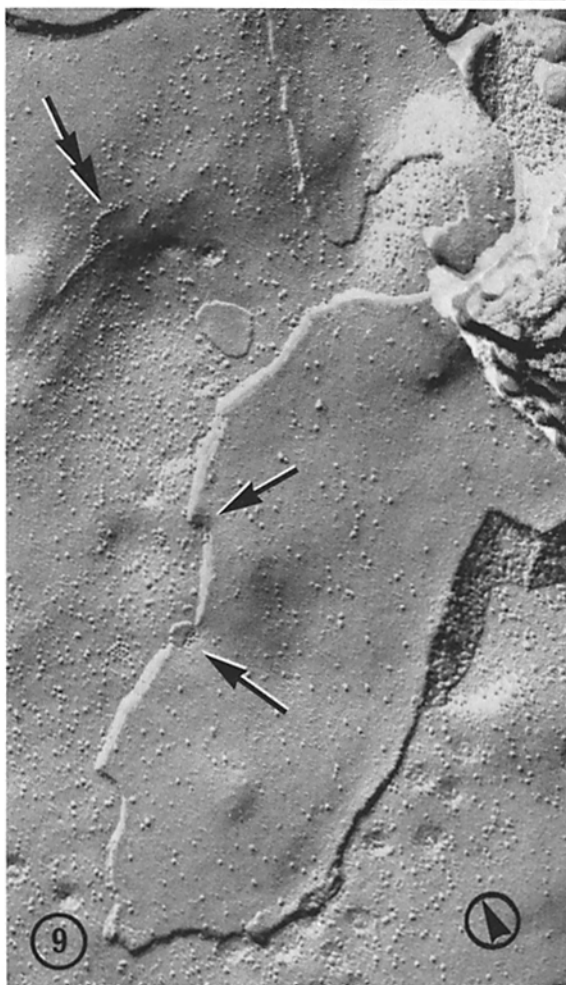
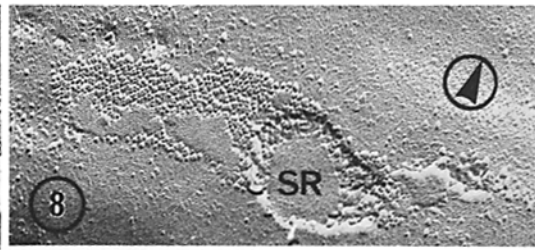
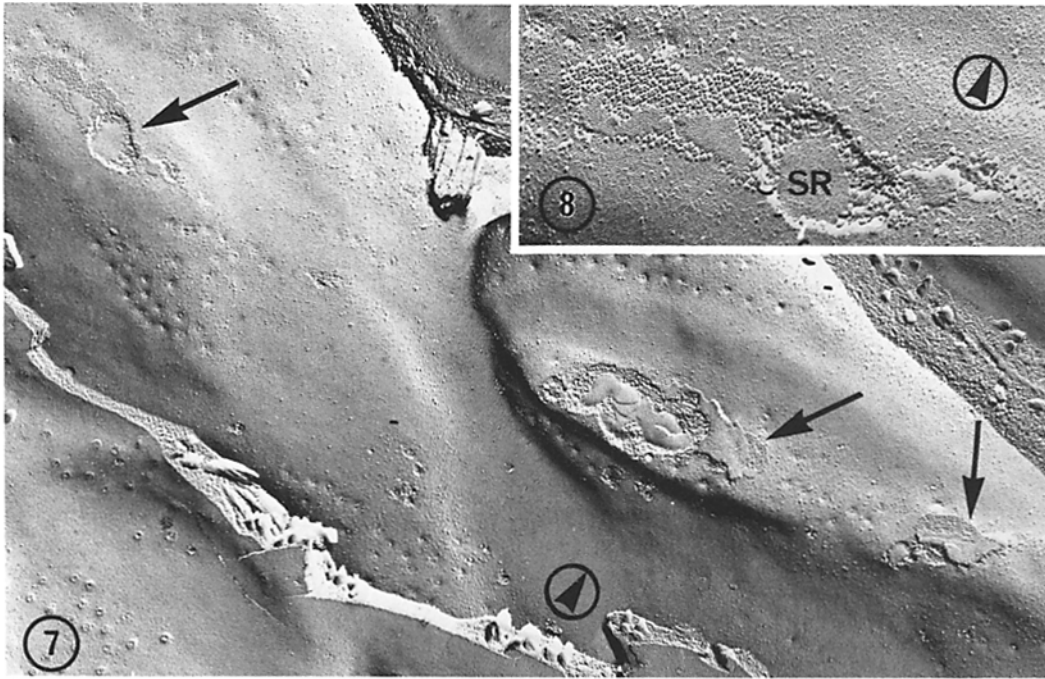
It is tentatively suggested that the small groupings of particles at regions where adjacent cells are closely apposed in freeze-fracture replicas may represent regions of cell to cell communication. In sectioned material, the presence of regions of close apposition without typical nexuses could represent regions of cell to cell communication, but, due to the limitations of present techniques, such a region cannot be conclusively demonstrated to be a junction. If regions of close apposition do correspond to a nexus and allow electrical coupling between smooth muscle cells, then in many blood vessels both innervated and noninnervated smooth muscle cells may be electrically coupled (9, 10).

A close relationship between some nexuses and an underlying cisterna of the sarcoplasmic reticulum was revealed in the present study of the guinea pig sphincter pupillae, chicken gizzard, and toad stomach. This relationship was noted also in sectioned sphincter pupillae (20) and resembles the subsynaptic cisternae described in this tissue by Uehara and Burnstock (36). A consistent relationship between nexuses and smooth surfaced endoplasmic reticulum has also been noted in mouse lutein cells during pregnancy, in freeze-fractured and sectioned material (1), where it was suggested that such relationships might be concerned with coordination of cellular synthetic activity. Since calcium has now been clearly demonstrated in sarcoplasmic reticulum in smooth muscle (29, 34), it is tempting to speculate that the presence of a large component of sarcoplasmic reticulum just beneath the nexus may control excitation-contraction coupling at this specialized junction. Since high intracellular calcium levels lower junction permeability (24), the junctional sarcoplasmic reticulum may act as a calcium sink and facilitate junctional permeability.

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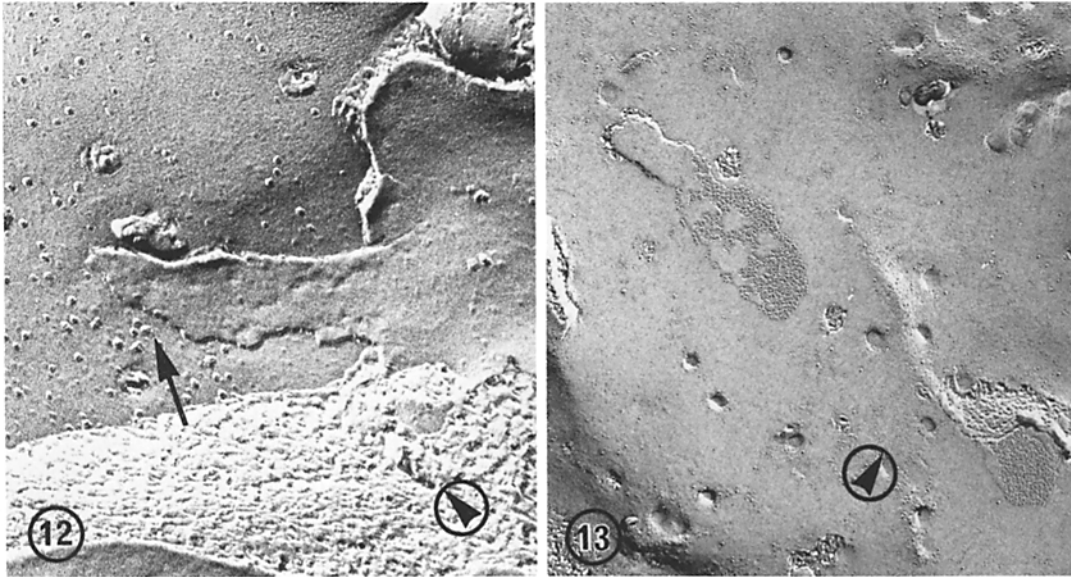


FIGURE 12 An example of a close membrane apposition in chicken anterior mesenteric artery smooth muscle cell. Although particles are present (arrow) and the two apposing membranes are close, there is no characteristic hexagonal array of a nexus. $\times 76,300$.

FIGURE 13 Typical nexus regions from the toad stomach. PF face particles are readily seen, but EF face pits are obscured. $\times 51,840$.

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FIGURE 7 Low-power view of the surface of chicken gizzard smooth muscle showing three nexuses (arrows) on the same smooth muscle cell. $\times 21,800$.

FIGURE 8 High magnification view of one of the nexuses shown in Fig. 7. There is a hexagonal array of particles (7-10 nm in diameter) with a spacing of 12-15 nm. Chicken gizzard. $\times 54,500$.

FIGURE 9 Guinea pig taenia coli smooth muscle showing groupings (single arrows) and rows of particles (double arrow) suggestive of nexuses. $\times 44,800$.

FIGURES 10 and 11 Guinea pig pulmonary artery showing a small grouping of particles suggestive of a nexus (Fig. 10) contrasting with an extensive area of particles (Fig. 11) which constitute a definite nexus. Fig. 10, $\times 108,000$; Fig. 11, $\times 100,000$.

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