Transactions of the Illinois State Academy of Science
(1983), Volume 76, 1 and 2, pp. 29-41

Nexuses in Intestinal Smooth Muscle: The Use of a Simple Tracer Technique

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ABSTRACT

Nexuses or gap junctions occur frequently between smooth muscle cells of the inner circular layer of the muscularis of the rat duodenum but apparently are not present in the outer longitudinal layer. While nexuses are visible and some details of structure can be seen in tissue routinely fixed and stained for electron microscopy, after lanthanum chloride treatment nexuses are electron dense and clearly visible at low magnifications. At higher magnifications, nexuses sectioned in all planes are identifiable whereas, in the main, nexuses in control material only are recognizable when cut perpendicular to the plane of the contact area, i.e. in nexuses showing a pentalaminar structure. In oblique and tangential ("en face") sections, connexons or particules are outlined by the lanthanum tracer and density and arrangement of packing of these particles is defined. The significance of these findings is discussed in relation to current knowledge of the nexus in smooth muscle and the gap junction in other cell types, and it is concluded that simple tracer techniques such as that utilized in this study still should prove useful in further studies of this type of cellular junction.

INTRODUCTION

One type of intercellular junction, the gap junction (or nexus), is concerned mainly with cell-to-cell communication and is believed to include intercellular

channels that mediate electronic coupling and the transport between cells of ions and small molecules (Bennett and Goodenough, 1978). Gap junctions are widely distributed in smooth muscle and other tissues and have been studied extensively by routine electron microscopy. Freeze fracture studies also have proved useful, particularly in respect of the surface distribution of the junctions, their shape and extent. In routine electron microscopy, gap junctions appear as areas of close apposition of the plasmalemmae of two adjacent cells with a slender gap of 2 to 3 nm between the membranes. While some intercellular tracers, such as lanthanum, penetrate this slender intercellular gap, the space is impermeable to other tracers such as horseradish peroxidase (Hopkins, 1978). When penetrated by such tracers the space also shows discrete bridges passing across the gap from one membrane to the other (Gabella, 1979). In sections where the plane of section is tangential to the cell surface and passes through a gap junction, 7 to 8 nm particles can be seen associated with the plasmalemmae, the particles often in hexagonal array. High resolution and X-ray diffraction studies have suggested that each particle or connexon unit is composed of six subunits forming a short cylinder with a central hydrophilic channel, these components of the opposed plasmalemmae being in register so that their hydrophilic channels are in continuity. Recently, a study of detergent-isolated gap junctions of mouse liver (Baker et. al., 1983), using negative staining with uranyl acetate, minimal irradiation methods and optical diffraction, has suggested that there are asymmetries in the gap junction with skewing of the six-lobed connexons. While all connexons may be identical chemically, their packing in hexagonal arrays on the two sides of the junction appeared to be nonequivalent.

In smooth muscle, gap junctions usually are called nexuses. Studies of intestinal smooth muscle to a great extent have been concerned with electrical coupling. An extensive study of electrically-coupled smooth muscles (Daniel et. al., 1976) showed that nexuses were common in some types but rare or absent in others. In the longitudinal muscle of dog intestine examined by thin section and freeze-fracture, nexuses were absent and it was suggested that in this tissue nexuses cannot account for electrical coupling. The freeze fracture appearance of the nexus in smooth muscle of several sites in different animals has been compared (Fry et. al., 1977). Usually, nexuses are oval, about 0.15 um^2 in surface area and, in the guinea pig sphincter pupillae muscle, numbered in the tens rather than the thousands per cell. The authors further discussed the minimum size of a nexus and concluded that, by freeze fracture, some with small aggregations of particles would not be recognized as nexuses in thin section. Further, they described the nexuses as being arranged in rows parallel to the main axis of the cell, usually alternating with longitudinal rows of plasmalemmal vesicles, and, in several locations, with a close relationship with sarcoplasmic reticulum. An extensive report (Gabella, 1979) has discussed cell junctions in the smooth muscle cell in relation to structural aspects of contraction. The author commented that nexuses in smooth muscle conform to the general structure reported for other tissues and confirmed previous reports that the number of nexuses varied considerably in different muscle. The sphincter papillae of the guinea pig shows numerous nexuses (Gabella, 1974; Fry et. al., 1977), as does the circular musculature of its ileum, where nexuses occupy about 0.2% of the cell surface (Gabella and Blundell, 1979). In the longitudinal muscle coat of taenia coli, nexuses are reported as small and few (Fry et. al., 1977), or extremely rare (Gabella, 1976). While in the cat small intestine nexuses are present in both layers of muscle (Taylor et. al., 1977), they are absent in longitudinal muscle of

dog duodenum (Henderson et. al., 1971), dog stomach (Daniel et. al., 1976) and guinea pig ileum (Gabella and Blundell, 1979). Gabella (1979), also suggested that in some tissues electrical coupling is achieved by a mechanism not involving gap junctions.

Gabella and Blundell (1979) studied the circular musculature of guinea pig ileum by freeze fracture to analyze quantitatively the nexuses between its smooth muscle cells. They reported an average cell surface area of 5,074 um^2 with a packing density of nexuses of 48 per 1000 um^2 of cell surface or about 244 nexuses per cell, a figure higher than an earlier estimate by Fry et alia (1977) for the sphincter pupillae muscle. Gabella and Blundell (1979) also described nexuses as varying in area from less than 0.1 to about 1.5 um^2, occupying about 0.212% of the cell surface. They found an average packing density of intramembrane particles (connexons) or complementary pits of about 7,200 per um of nexal surface and extrapolated this as indicating that there may be up to 77,000 intercellular channels within the total complement of nexuses of a single muscle cell. In a further study, Gabella (1979) produced muscle hypertrophy in guinea pig ileum by experimental stenosis and found an increase in size and number of cells, with gap junctions larger than in control animals. However, while cells doubled their surface area, the number of nexuses per unit area remained unchanged.

As indicated above, gap junctions are widely distributed throughout the animal kingdom (Gilula, 1977). Indeed their absence in animal tissues is exceptional. They do not normally occur between circulating blood cells nor between mature skeletal muscle cells, where they have been rendered redundant by cell fusion into a syncytium, and do not occur between neurones and glial cells (Bennett and Goodenough, 1978). In all sites, they have a similar, if slightly variable, appearance as described above. Recently, studies on gap junctions or nexuses have involved techniques such as freeze fracture diffraction, negative staining and biochemical methods and little attention has been paid to older methods. The junctions were described first by routine electron microscopy but it was the work of Revel and Karnovsky (1967) that added much to our knowledge of junctional structures. They used the colloidal lanthanum tracer technique that permitted visualization of the gap junctional lattice in cardiac and liver tissue. Recently, a study of duodenal enterocytes compared basic aldehyde fixation with results using Malachite Green or lanthanum chloride (Leeson and Higgs, 1982). The results suggested that the addition of lanthanum to the fixative was valuable and recent work on lanthanum chloride as a marker for intercellular junctions in rat exocrine pancreas has been reported also (Leeson and Leeson, 1982). The purpose of the current study is to demonstrate the use of this lanthanum tracer technique in investigating gap junctions of smooth muscle in the muscularis of the rat duodenum.

MATERIALS AND METHODS

After anesthetization with ether, thoraxes of twelve young adult rats (50 to 120 days) were opened and the animals perfused via the left ventricle with a fixative containing glutaraldehyde, 3%, and formaldehyde, 3%, in 0.2M cacodylate buffer at pH 7.2, and 10mM with respect to lanthanum chloride (Sigma Chemical Company). A further six rats as controls were treated similarly except the basic fixative without lanthanum chloride was used. In all animals, the duodenum was removed, minced and placed in fresh fixative for two hours, washed in buffer,

dehydrated through graded ethanols, and infiltrated and embedded in an Epon-Araldite mixture, the tissue oriented as far as possible to permit either transverse or longitudinal sectioning. Ultrathin sections (silver grey) were cut on a Porter Blum II ultramicrotome and supported on 300 mesh grids. Sections were examined without further staining, or after staining either with alcoholic uranyl acetate for 10 minutes or lead citrate for three minutes, or both. Electron microscopy was performed with a Philips EM 200 at 60 KV or an EM 410 at 80 KV.

RESULTS

In all the material examined, no nexuses between muscle cells were seen in the outer longitudinal coat of the duodenal muscularis although nexuses were frequent in the inner circular layer. In control material, i.e. in material not exposed to lanthanum chloride, nexuses were seen with difficulty in unstained sections where generally they only were visible as 'linear' profiles, i.e. as pentalaminar structures. Where sectioned in planes other than perpendicular to the nexus, they proved very difficult to identify. In stained sections of control material, nexuses sectioned in all planes were identifiable in the circular layer, although the great majority was the linear profile. In lanthanum treated materials, nexuses were markedly electron dense and easily seen even in unstained sections, although staining enhanced contrast of the tissue components. Section staining in this material apparently caused no change or improvement in appearance or contrast of the nexus. However, most illustrations are from stained material as overall tissue contrast and appearance were enhanced.

Nexuses varied in appearance and extent, presumably largely due to the plane of section. Some appeared extensive, others extremely small. In many cells, no nexus was seen in the section while others showed such contacts with one or more adjacent cells. Frequently, more than one nexal contact was made with another cell and occasionally a nexus occurred between two processes of the same cell. Nexuses were seen in all areas of the cell, i.e. in the nuclear region as well as the cytoplasmic extensions of the cell, and were formed by contacts between cell bodies or cell processes. In form, they varied with the plane of section from linear (from straight to curved) to oblique to tangential, i.e. cut through the nexus parallel to its surface, the thickness or "width" of the resultant profile correspondingly increasing. As the plane of section varied, so did the morphology of the nexus and these appearances combined made possible a three-dimensional interpretation.

In control material at low magnification, smooth muscle cells of both layers of the muscularis generally showed a close packing with little intercellular material and very few other cells, mostly fibrocytes. Between the two layers, intercellular material was more prominent with collections of ganglion cells and nerves of the intermyenteric plexus frequently seen. Small nerve bundles and occasional ganglion cells were seen between smooth muscle cells in each layer, more frequently in the peripheral zone of the circular layer. The junction between the two muscle layers was clear, as was the junction between the submucosa and the inner circular layer. Intermediate and gap junctions between muscle cells of the circular layer were identifiable at low magnification. At higher magnifications, nexal contacts in the main appeared as linear profiles, varying in extent and location, i.e. some occurring between cell surfaces, other between one cell and a process from another cell, or between processes from adjacent cells. The pentalaminar appear-

ance of these linear profiles was seen clearly at high magnification, the central dense lamina in some being continuous, in others interrupted and, where cut slightly obliquely, with dense transverse lines. Oblique and tangential sections through nexuses in this material proved difficult to identify and little detail in them was discernable. In cell cytoplasm adjacent to nexuses, small vesicles or caveolae and mitochondria were seen frequently.

In lanthanum treated material at low magnification, nexuses were seen easily due to their enhanced electron density regardless of grid staining or not. As in control material, their number, form and location varied widely. In many cases, in the inner circular layer the majority of cells showed at least one nexus around its circumference and many cells showed more than one. In a few instances, a nexus was present between a smooth muscle cell and an element of nerve tissue. While nexuses were absent in the longitudinal layer, muscle cells there showed numerous subsarcolemmal caveolae, these caveolae also being present in cells of the circular layer but in fewer numbers. The frequency and clarity of visualization of nexuses with lanthanum was demonstrated also at medium power with several such contacts between adjacent cells and, frequently, contacts between three cells or their processes.

At higher magnifications of nexuses showing basically a linear or pentalaminar appearance, there was some variation in appearance. In thicker sections, or where lanthanum penetration had been extensive, the central electron-dense lamina appeared thichened to about 4 nm and complete, with regularly spaced dense transverse lines passing across the junction where the plane of section was slightly oblique. In thinner sections, or where lanthanum penetration was less dense, the central dense lamina appeared interrupted at regular intervals. Often, a nexus showed at one extremity the pentalaminar structure with, at the other, a pattern of transverse lines, sometimes grading to an apparent pattern of circular profiles, this presumably resultant upon the plane of section. In more tangential or 'en-face' sections, further detail was apparent. In such sections, the nexus showed a pattern of small circular, low density profiles of 8 to 9 nm diameter arranged in hexagonal array, with dense lanthanum deposit around and between these profiles. In a few instances, the circular profiles showed a small, central dense dot. These morphological features of the nexus were also visible in unstained but lanthanum treated material. Also, in just two micrographs, lanthanum in addition to infiltrating a nexus also filled one or more small vesicles that communicated by necks with the intercellular space.

DISCUSSION

The history of our knowledge of gap junctions is relatively short but their form, distribution and function have aroused considerable attention in recent years. A hexagonal packing of subunits associated with plasma membranes after permanganate and osmium tetroxide fixation was described by Robertson (1963) in electrical synapses of Mauthner cells, and a similar pattern in negatively stained isolated hepatic cell plasma membrane junctions was illustrated by Benedetti and Emmelot (1965). Revel and Karnovsky (1967) first described the use of lanthanum salts to demonstrate a hexagonal array of subunits in intercellular junctions of mouse heart and liver and their report includes illustrations remarkably similar to those of this study. It was the study by Revel and Karnovsky that first distinguished

between true tight junctions (zonulae occludentes) and gap junctions and they observed that the hexagonal lattice outlined by the tracer material lay within the 2 nm gap. Later, it was shown that the nexus of smooth muscle also showed a 2 nm gap and a hexagonal lattice demonstrable by colloidal lanthanum (Revel, Olsen and Karnovsky, 1967). While other workers have used this technique since that time, most recent studies of the smooth muscle nexus and gap junctions in other tissues have utilized freeze fracture and other techniques. The current study reaffirms the value of a simplified lanthanum technique. This is not to deny the value of other techniques that have clarified and extended our knowledge and understanding of the gap junction.

The morphological detail revealed by using lanthanum is much greater than that after routine fixation and staining. Indeed, in intestinal smooth muscle this study confirms that nexuses are not seen easily and that the great majority are of the 'linear', pentalaminar type. A tangential section of a nexus apparently is not recognizable as such although Robertson (1963) did demonstrate them in permanganate-fixed tissue. In comparison, after lanthanum treatment, nexuses are identified easily at low magnifications which facilitates studies of their number, size and distribution, and at higher magnifications, nexuses cut in all possible planes are visible and details of structure are seen, varying with the plane of section. Indeed, while studies of smooth muscle using freeze fracture have given much information (Fry et. al., 1977: Gabella and Blundell, 1979: Gabella, 1979), similar information has been obtained with the much simpler tracer technique. Other techniques provide different and, often, additional information. For example, Raviola et. al, (1980), studied gap junctions in several non-muscle cells with freeze fracture after rapid freezing and demonstrated that they are highly pleomorphic in the living state, this perhaps accounting for the variations in structure reported after chemical fixation. As Peracchia and Peracchia (1980) have pointed out, there now is evidence that intracellular cations, particularly free calcium, have a role in the mechanism of cell uncoupling whereby direct cell to cell communication via gap junctions is interrupted. They studied such junctions isolated from lens fibers and exposed to a pH of 6.5 or lower to establish the role of H^+ in this process. They found that the freeze fracture appearance of both control and low pH treated junctions was not altered by glutaraldehyde fixation and cryoprotective treatment as had been suggested previously (Heuser et. al., 1975) in a study where intact and isolated junctions were freeze fractured after rapid freezing. At low pH, Peracchia and Peracchia showed that particles (connexons) in the gap junction most often form orthagonal and rhombic arrays. Recently, in mouse liver gap junctions isolated with detergents and negatively stained with uranyl acetate, Baker et. al. (1983) used low-irradiation methods for recording and showed that substructural features of the junction are acutely sensitive to irradiation. Their method revealed, in the hexagonal junction lattice, skewed hexameric connexons, suggesting that the two hexagonal membrane arrays that form the junction may not be structurally identical. Thus, while obviously valuable, the lanthanum tracer technique has its limitations.

Studies that have reported on the size and number of gap junctions and the packing density of particles of the gap junction as seen in freeze fracture preparations (Fry et. al. 1977; Gabella, 1979; Gabella and Blundell, 1979) have shown some variation, perhaps due to variation in cell and species types, but variations also occur with physiological state. In guinea pig ileum, nexuses of the circular

muscle layer occupy 0.212% of the cell surface and in each cell there may be up to 77,000 intercellular channels in the total nexus complement (Gabella and Blundell, 1979). After the production of hypertrophy following experimental stenosis, gap junctions increase in size and occupy 0.49% of the cell surface (Gabella, 1979). Differences in gap junction size between cultivated normal and hypertensive smooth muscle cells of the rat aorta have been studied by freeze fracture (Grünwald et. al, 1982). These investigators found that the total surface area of plasma membrane occupied by gap junctions was much smaller in normal than in hypertensive smooth muscle and concluded that the results indicate an intense metabolic communication between cells in the hypertensive smooth muscle cell cultures. Other cell types show changes in gap junctions with physiological state. For example, exogenous estrogen stimulation promotes gap junction growth indirectly in ovarian granulosa cells while exogenous FSH stimulation directly amplifies the developmental sequence of gap junction growth and turnover (Burghardt and Matheson, 1982). Several studies have been performed on uterine smooth muscle. Reports that bilateral section of the pelvic parasympathetic nerves in the rat block parturition led to a study of myometrial gap junctions (Burden et. al, 1979). These investigators found a significant increase in the number of gap junctions by day 23 in the animals after pelvic neurectomy and concluded that the parturition block is not attributable to failure of gap junction formation in smooth muscle cells. Garfield and Hayashi (1980) suggested that gap junctions between myometrial cells in nonpregnant women may form in response to physiological and pathological stimuli such as the production of prostaglandins and that the presence of these intercellular contacts may result in contractions of the uterus. In a subsequent study (Garfield and Hayashi, 1981), the same investigators studied myometrial tissue in various stages of labor and found increasing gap junctions with increased cervical dilatation or increased frequency of uterine contractions. They proposed that gap junction formation may be stimulated by some physiologic change and that the appearance of gap junctions may terminate pregnancy by resulting in coordinated, synchronized muscle activity and dilation of the cervix. In a study in guinea pigs, gap junctions in myometruim were found to increase at parturition (Garfield et. al., 1982), suggesting that an increase in myometrial gap junction area is associated with, and may be essential for, parturition. In chicken gizzard smooth muscle over several developmental stages, La Manita and Shafiq (1982) using the freeze fracture technique found not only an increase in intramembranous particles per gap junction but also an increase in particle density. Thus, in several tissues, gap junctions show changes with age, and with physiological and pathological state. Such studies of size, number, packing density could be undertaken using the lanthanum tracer technique, although one report (Fry, et. al., 1977) has suggested that small nexuses may not be recognizable as such in thin sections.

As stated earlier, many studies of the nexuses of intestinal smooth muscle have been concerned with electrical coupling (Daniel et. al., 1976) and this study has confirmed previous reports of the sparsity or absence of nexuses between muscle cells of the outer longitudinal layer (Fry et. al, 1977; Gabella and Blundell, 1979; Henderson et. al, 1971; Daniel et. al, 1966; Gabella, 1979). Among others, Gabella (1979) has suggested that electrical coupling is achieved by a mechanism not involving gap junctions. In an interesting study of the connective tissue of longitudinal and circular muscle of the rat small intestine, Taylor et. al, (1977)

observed electrotonic spread of applied potentials between the muscle layers with no rectification, and found nexal junctions between muscle cells of both layers. Since connective tissue can serve for electrical conduction between cultured heart cells and since electrical properties of intestinal muscle permit transmission with low degrees of coupling, they suggested that interstitial cells and fibrocytes may electrically couple both muscle layers in the rat intestine. This is an interesting hypothesis that merits further study.

CONCLUDING REMARKS

This study using a simplified tracer technique has shown that much useful information can be obtained concerning the location, number, size and general distribution of nexuses in intestinal smooth muscle, and some information about the structure of the nexus in respect of particle (connexon) size and packing. The results have been discussed in relation to current knowledge of the nexus/gap junction, its morphology, functions and variations that have been reported with changes in methodology and physiologic and pathologic state. While our knowledge of the gap junction has improved steadily over the past decade and a half, much work remains to be done, for example, even in the area of the correlation between morphological studies in intestinal muscle and the apparent species variation in relation to electrical coupling. It is suggested that in future studies, the use of simple tracer techniques should not be ignored as they still can facilitate such investigations.

DESCRIPTION OF ILLUSTRATIONS

Plate I

Fig. 1. Muscle fibers (L) of the outer longitudinal layer of the muscularis of rat duodenum are seen at lower left and show no nexuses in this section treated with lanthanum and stained with lead citrate and uranyl acetate. Muscle fibers of the inner circular layer occupy the remainder of the field and show numerous, electron-dense nexuses (arrows). Also seen are a small nerve (N) and blood vessel (V). x 3,500.

Fig. 2. An unstained section from a control (non-lanthanum) rat showing a process (P) of a muscle cell of the circular layer forming nexuses with two other cells (lower left, top right). Both nexuses show a pentalaminar structure with deficiencies in the central dense lamina (arrowheads) indicating connexons. x 115,000.

Fig. 3. An extensive nexus from a lanthanum treated, unstained section showing the pentalaminar structure cut transversely, with regular deficiencies in the central dense lamina, but showing regular, transverse linear densities (arrows) where cut slightly obliquely, these indicating lanthanum penetration between connexons. x 105,000.

Plate II

Fig. 4. Portions of three muscle fibers (1, 2, 3) are seen with linear nexal contacts (top and bottom) densely stained by lanthanum and with thick, continuous central dense laminae. The central nexus involves all three cells and is sectioned obliquely with little detailed structure apparent. The section was block-stained. x 52,000.

Fig. 5. A nexus here is cut transversely, from lanthanum and double stained material. The pentalaminar structure is seen with a dense central lamina, mainly continuous but with some interruptions (arrow). x 85,000.

Fig. 6. This section, from block-stained, lanthanum treated material, shows two nexuses between three muscle cells, the one on the left cut transversely and showing the pentalaminar structure, the one on the right cut obliquely and showing a pattern of electron lucid, small circular profiles (connexons) (arrow) in electron-dense material. x 74,000.

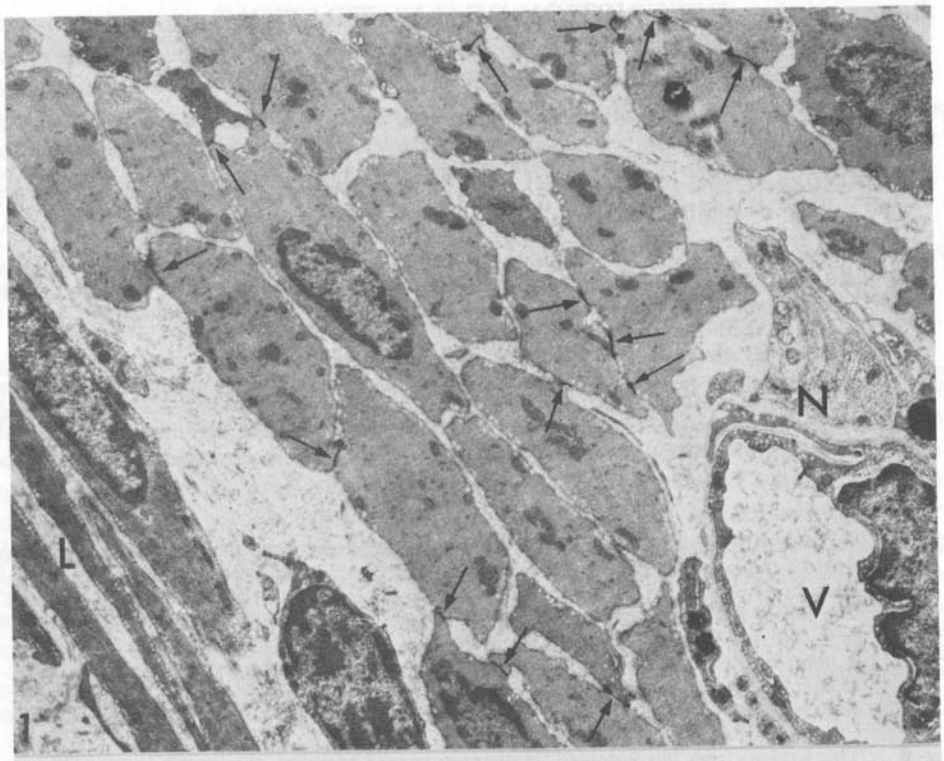
Fig. 7. This S-shaped nexus is from unstained, lanthanum treated material. In this 'en-face', tangential section through the center of the nexus a regular hexagonal pattern of electron lucid particles or connexons is seen, lying in electron dense material (arrows). Centrally, where the plane of section is oblique, this pattern is not seen (asterisk). x 115,000.

Plate III

Fig. 8. In this unstained but lanthanum treated material a muscle cell (M) makes nexal contacts (left) with two other processes of muscle cells (P), here sectioned nearly transversely but showing a thick central dense lamina. At right, a further nexus (arrow) is cut 'en-face' and shows a regular hexagonal pattern of electron lucid connexons with dense material surrounding them. x 74,000.

Fig. 9. Similar to Figure 8, but double stained and showing a nexus 'en-face' with pale particles or connexons in hexagonal array, some with a small, central dark dot (arrowheads) indicating the central channel of the connexon. x 210,000.

Fig. 10. A nexus between two muscle cells is seen, showing at top and bottom the pentalaminar structure with a continuous, dense central lamina but centrally the two plasmalemmae separate and in this area two small vesicles or caveolae (arrows) show continuity with the extracellular space and contain dense material. The material was double stained and lanthanum treated. x 115,000.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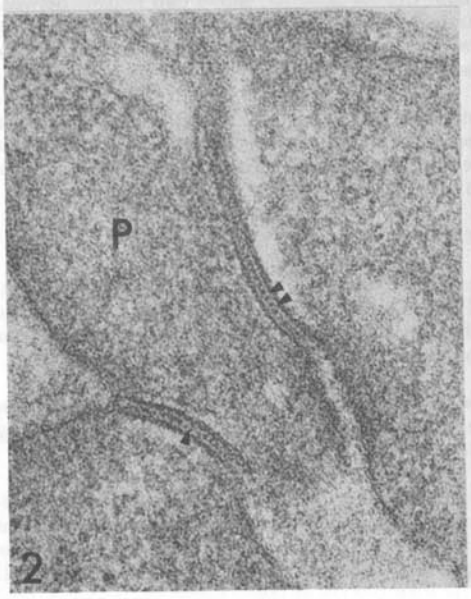
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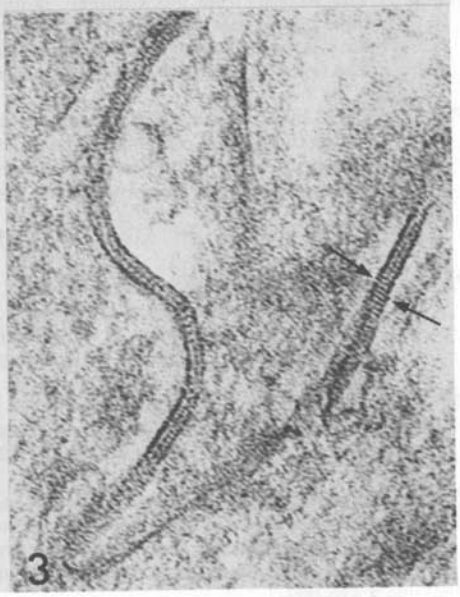
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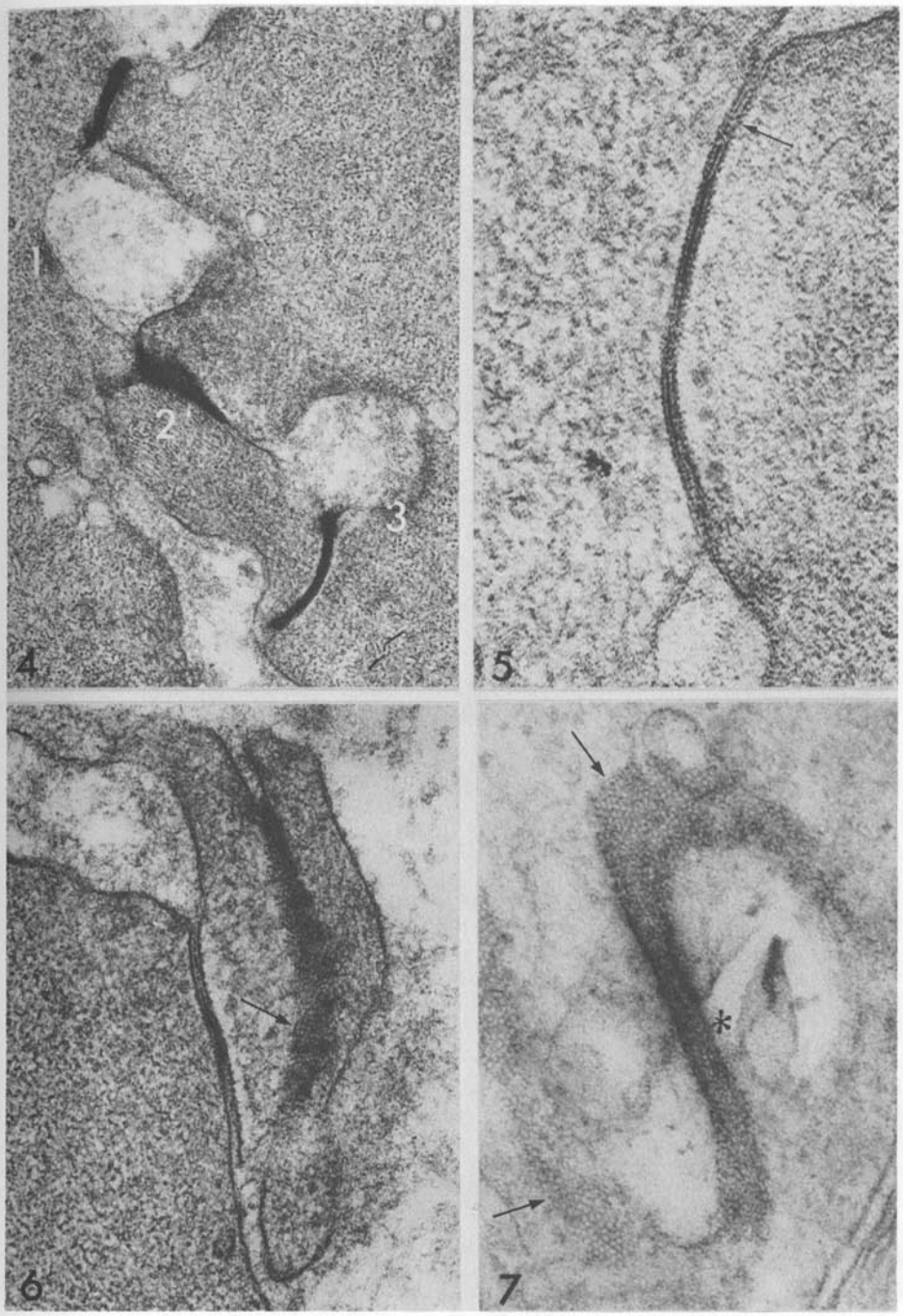
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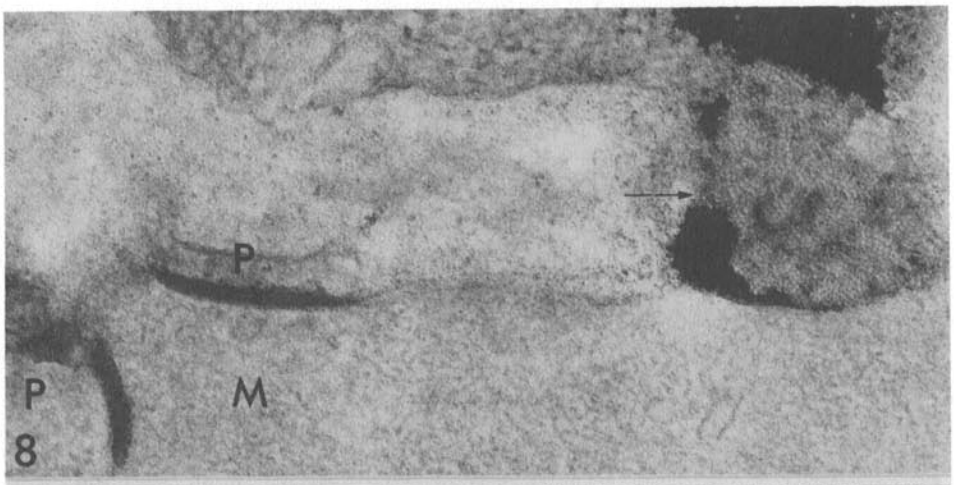
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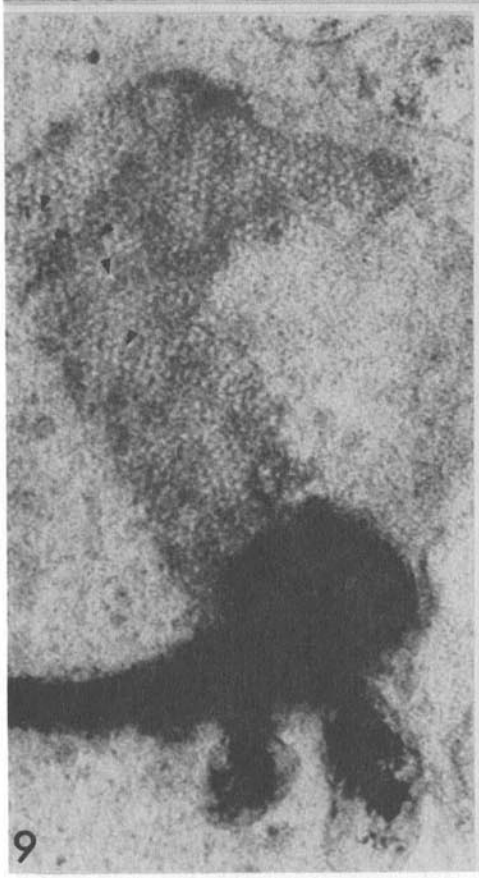
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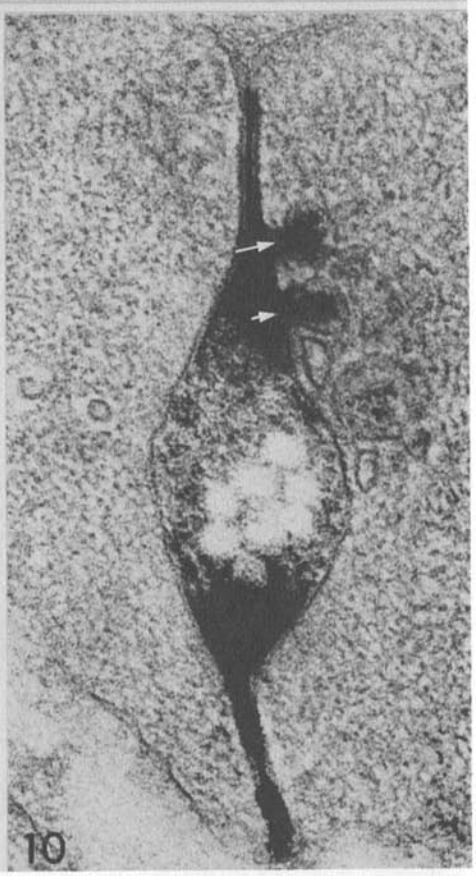
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