

NEXUSES BETWEEN THE SMOOTH MUSCLE CELLS OF THE GUINEA-PIG ILEUM

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ABSTRACT

The circular musculature of the guinea-pig ileum has been studied by freeze-fracture to analyze quantitatively the gap junctions (nexuses) between its smooth muscle cells. The average cell surface area and cell volume are $5,074 \mu\text{m}^2$ and $3,260 \mu\text{m}^3$. The packing density of nexuses is $48/1,000 \mu\text{m}^2$ of cell surface or ~ 244 /muscle cell. Nexuses range in area from <0.1 to $\sim 1.5 \mu\text{m}^2$ and they occupy 0.212% of the cell surface. The average packing density of intramembrane particles or pits in nexuses is $\sim 7,200/\mu\text{m}^2$ of nexal surface, indicating that there may be $\sim 77,000$ intercellular channels in the full complement of nexuses of one muscle cell.

KEY WORDS nexus · gap junction · smooth muscle · intestinal musculature · electrical coupling · freeze-fracture

Nexuses between smooth muscle cells were described over 15 years ago in a variety of muscles (6, 7). By the use of lanthanum as an extracellular tracer, it was later shown that nexuses between smooth muscle cells are "gap junctions" (23, 27). They are readily identified as areas of intimate apposition between the membranes of adjacent cells, and, in freeze-fracture preparations, as characteristic clusters of intramembrane particles (see reviews in references 13, 19, 22). It has generally been assumed that nexuses represent the sites of electrical coupling between smooth muscle cells (1, 2, 6). This notion, however, may require some revision in view of the reports that there is not always a good correlation between the degree of electrical coupling and the presence of nexuses. The observation that some smooth muscles with good cable properties show few or no nexuses suggests that there may exist other structural correlates of electrical coupling in addition to the nexuses (see a review in references 5, 16). Much more experimental evidence is needed to clarify

this problem. It is also particularly important to have accurate quantitative estimates of the nexuses in those muscles that are known to be well supplied with them. This prompted us to investigate by freeze-fracture the nexuses of the circular musculature of the guinea-pig ileum. Relatively few freeze-fracture studies have been published on nexuses in smooth muscles (8, 12, 28), and only one of them examines the distribution of nexuses in a variety of smooth muscles (8).

MATERIALS AND METHODS

Male albino guinea-pigs (body weight 350–500 g) were used. They were killed by cervical dislocation and exsanguinated. The full length of the small intestine was excised and put in oxygenated Krebs' solution. Portions of the ileum 80–200 mm from the ileo-colonic junction were gently distended by injecting Krebs' solution into the lumen and ligating the ends. The tissue was then immersed in fixative (5% glutaraldehyde in 0.1 M Na cacodylate at pH 7.4 with 0.04 M CaCl_2) at room temperature for 2–3 h, during which time it was cut into small rings. At the end of the fixation, after 15 min in several changes of buffer alone the specimens were immersed for 1 h in 15% glycerol in cacodylate buffer, followed by 1 h in 25% glycerol. Under a dissecting stereomicroscope, the rings were slit open and mucosa

and submucosa were peeled off. The rest of the wall (serosa and muscularis externa) was cut into rectangles of known orientation, measuring $\sim 1 \times 1.5$ mm, and these were mounted on support discs and frozen in liquid Freon 22 (ISE Chemical Ltd., Bristol, England) cooled to -150°C on liquid nitrogen. Freeze-fracturing was carried out in a Balzers BAF 300 apparatus (Balzers Corp., Nashua, N. H.). The material was fractured at -105°C and immediately shadowed with platinum-carbon at 45° followed by carbon at 90° . The tissue was removed in Na hypochlorite and, after cleaning in double-distilled water, the replicas were collected on 200-mesh uncoated grids.

The replicas were examined in a Phillips 300 electron microscope equipped with goniometer stage and rotating specimen holder. Satisfactory areas were photographed at $\times 10,000$ in order to prepare large montages; nexuses could not be recognized on the fluorescent screen at this magnification, and the areas were selected on the sole basis of absence of knife marks and reasonable cleanness. When the photography of the area selected for the montage was completed, the same area was scanned again at $\times 26,000$ or $\times 41,000$, and all the nexuses were photographed individually. The montages were made up with prints enlarged $2.5\times$. With the aid of a magnifying glass, the nexuses were identified, outlined, and individually numbered and referred to their photographs at higher magnification ($\times 26,000$ or $\times 41,000$, and photographically enlarged $2.5\times$).

On the montages, the areas to be used for nexus counts were selected by discarding: (a) areas that were technically inadequate (knife marks, contamination, insufficient shadowing, etc.); (b) the areas covering extracellular space; (c) the areas covering nonmuscle cells; (d) the areas covering the cytoplasm of muscle cells. The areas occupied by smooth muscle cell membranes and usable for nexus counts were outlined, transferred on tracing paper, and measured with a planimeter.

Areas covering the cell membrane of muscle cells were sometimes discarded because they appeared sloping instead of lying flat across the path of the electron beam. This was not a frequent occurrence, contrary to what we had anticipated considering the curvature of the smooth muscle cell surface. Probably many parts of the replica flatten down when the replica is collected on the copper grid. To make sure that the areas of the replica selected for measurement were flat, some nexuses were photographed after tilting the replica in the goniometer stage (usually $\pm 12^\circ$). Since, in the areas selected, the replica photographed at 0° of tilt was nearer the horizontal orientation than when photographed at either $+12^\circ$ or -12° , no correction of the directly measured area was introduced.

The number of intramembrane particles was obtained by projecting negatives at $\times 26,000$ or $\times 41,000$ through a Carl Zeiss (Jena) Dokumator (film reader), set at magnifications ranging between $\times 6$ and 17 ; from the projected nexus, the outline and all the pits or particles

were drawn in pen on drawing paper. The area was measured with a planimeter, and the number of pits or particles was counted with the aid of a pen-fitted culture-counter. The areas of the nexuses were also measured with a similar method on photographic enlargements of the montages.

RESULTS

In sections of the circular musculature of the guinea-pig ileum examined by conventional electron microscopy, nexuses between smooth muscle cells were often encountered (Fig. 1). By freeze-fracture, nexuses were identified as clusters of intramembrane particles (Figs. 2 and 3) or clusters of intramembrane pits (Figs. 2 and 5) according to the criteria published in the literature (13, 19, 22). Each intramembrane face at the level of a gap junction showed either exclusively particles (P face) (Figs. 3 and 4) or almost exclusively pits (E face) (Figs. 6 and 7) (only rarely was an isolated particle seen in a nexus exposed on the E face [Fig. 6]); when pits and particles were present in the same junction, it was clear that they belonged to superimposed membranes (Fig. 2). Intramembrane particles measured ~ 8.5 – 11 nm in diameter, and they were not perfectly uniform in size. The most common center-to-center distance was ~ 11 nm. In many nexuses, small patches or aisles devoid of particles or pits subdivided the particles and pits into smaller groups (Figs. 4, 5, and 6).

The shape of nexuses was variable (Fig. 7). The small ones tended to be round or ovoid, with the long axis parallel to the cell length. Larger nexuses were elongated, their length being up to five times their width, with their long axis almost invariably parallel or nearly parallel to the cell's length. Most nexuses had a smooth contour (Figs. 3, 4 and 6). In 3–5% of the nexuses, usually among those of large area, there was a small cluster of intramembrane particles or pits (3–35) situated only 10–100 nm away; we indicate this arrangement as a nexus with a satellite, and in the nexus counts it was counted as one unit (Fig. 5). We never observed caveolae within the area of a nexus but occasionally a row of caveolae may be lying fairly close to a nexus (Fig. 4). Sometimes a cisterna of the sarcoplasmic reticulum lay immediately beneath a nexus (Fig. 6), but this association between the two structures was rarer than has been observed in other smooth muscles (8, 9).

Large montages were used for the study of nexus number, and the figures shown in Table I were obtained. Over a total surface of $9,603 \mu\text{m}^2$ ex-

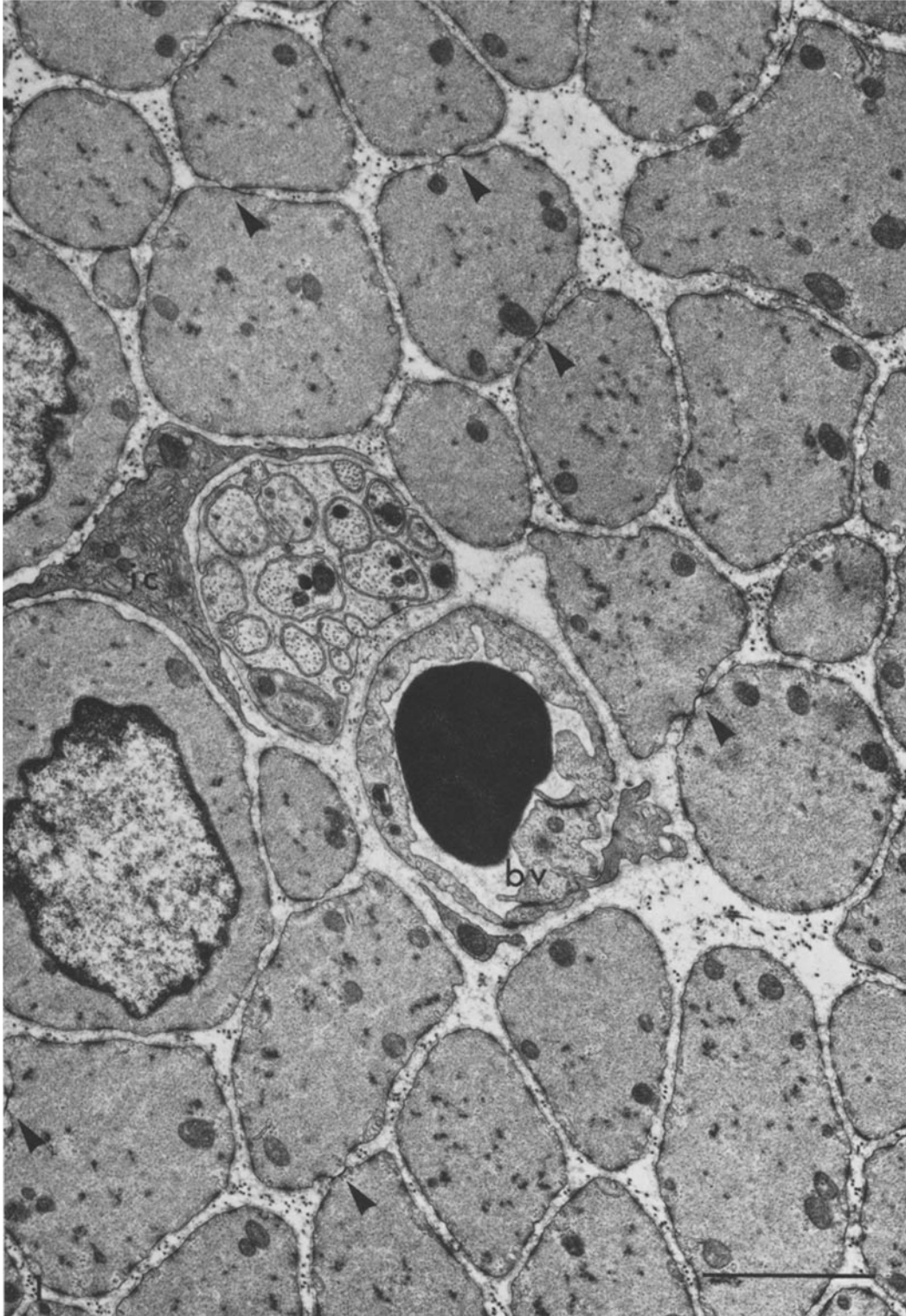


FIGURE 1 Transverse section of the circular muscle of the guinea-pig ileum. The smooth muscle cells are separated by an intercellular space with prominent collagen fibrils. Many bands of dense material are present at the inner aspect of the cell membrane and sometimes they match each other in adjacent cells. A number of nexuses are visible (arrowheads). (*bv*) a blood vessel; (*ic*) an interstitial cell closely related to a nerve. Bar, 2 μ m. \times 13,500.



FIGURE 2 Freeze-fracture preparation of two muscle cells whose E (top left) and P (bottom right) faces are exposed. The two cells show three nexuses in their area of contact while they are elsewhere separated by intercellular space (dark band). Another nexus is visible over the bottom right corner. Bar, 0.5 μm . \times 39,000.

amed, we counted 412 nexuses, or $48/1,000 \mu\text{m}^2$. The overall surface of all the nexuses counted amounted to $18.261 \mu\text{m}^2$, or 0.212% of the cell surface.

The size distribution of all nexuses examined in one experiment is shown in Fig. 8. They range in area from $<0.1 \mu\text{m}^2$ to $\sim 1.4 \mu\text{m}^2$, and over half of them are $<0.3 \mu\text{m}^2$ in area. No difference in size distribution between nexuses on the P face and on the E face was found.

Particles and pits were counted on 140 nexuses. The packing density of either of them ranged between 5,300 and $10,000/\mu\text{m}^2$. On an overall nexal surface of $7.246 \mu\text{m}^2$, we counted 52,140 particles and pits, indicating a mean packing density of $7,196/\mu\text{m}^2$. The mean of the packing densities of the individual nexuses was $7,263/\mu\text{m}^2$ (SD ± 981 , N = 140). When nexuses exposed on the P face (particles) and nexuses exposed on the E face (pits) were considered separately, on a nexal surface of $3.447 \mu\text{m}^2$ (74 nexuses) we counted 24,044 particles, i.e., $6,975/\mu\text{m}^2$, and on a nexal surface of $2,329 \mu\text{m}^2$ (43 nexuses) we counted 17,350 pits, i.e., $7,449/\mu\text{m}^2$.

As to the distribution of nexuses over the cell surface, this was very variable from one cell to another (Fig. 9). Presumably, some cell surfaces were facing the connective tissue of an intramuscular septum, and we therefore expected to find the whole exposed surface of some cells devoid of nexuses. On those cells that showed nexuses, their numbers were variable and we were usually not able to recognize any particular pattern in their arrangement (for example, in the distance between nexuses). Sometimes, however, nexuses appeared distributed along a line parallel to the cell's axis.

The area and volume of the muscle cells were calculated by the method described in reference 10 by transmission microscopy on sections of adjacent segments of the small intestine. The cell area was $\sim 5,074 \mu\text{m}^2$ and the cell volume $3,260 \mu\text{m}^3$.

DISCUSSION

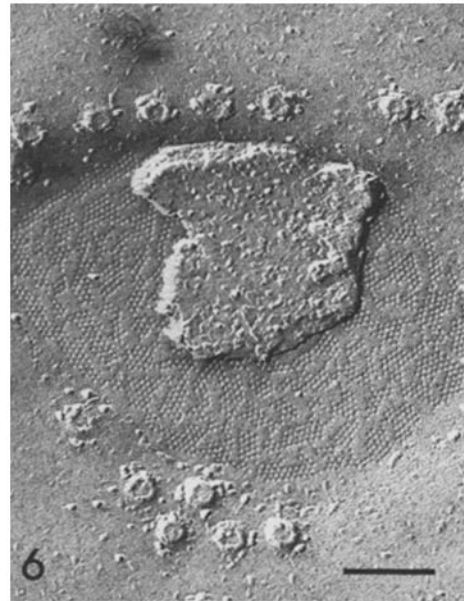
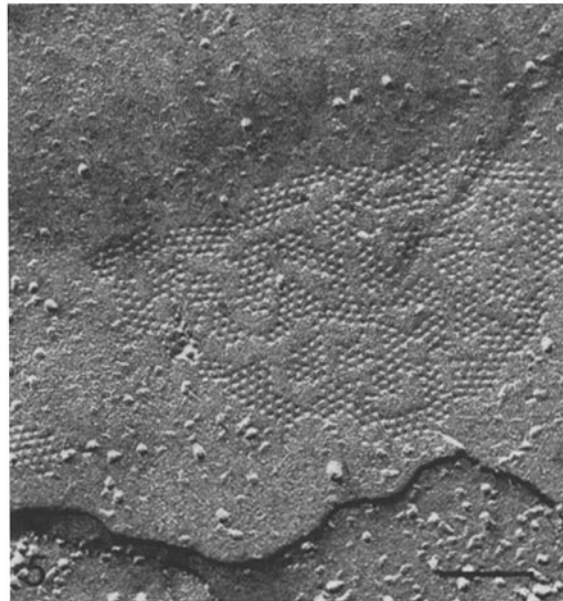
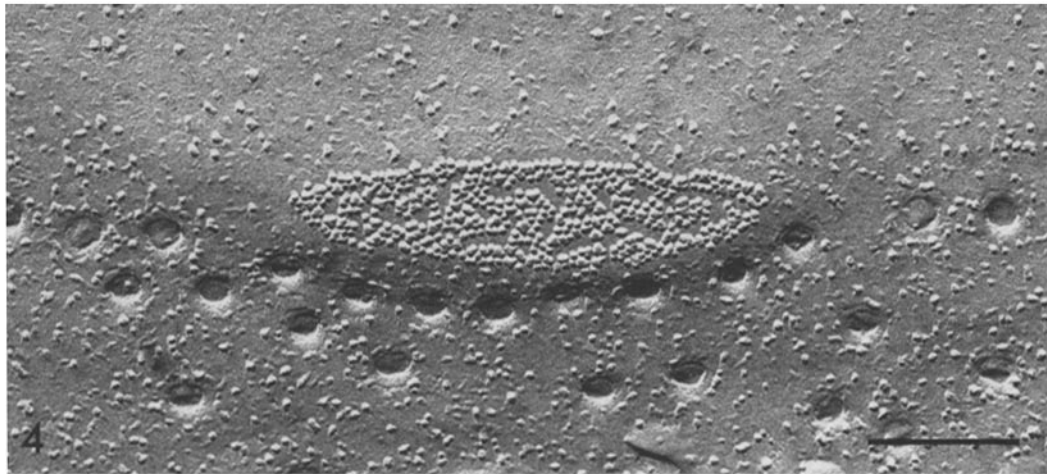
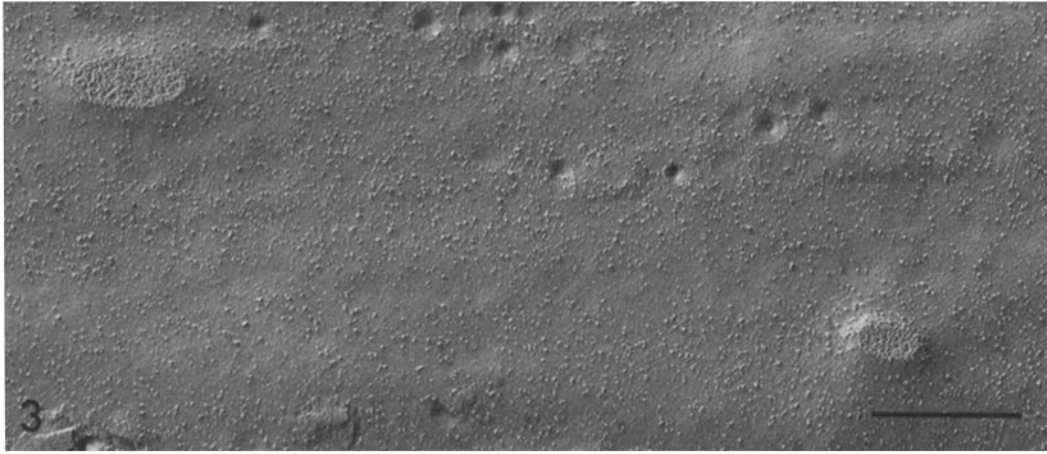
The present work is an attempt to provide a quantitative account of the nexuses in the circular musculature of the guinea-pig ileum. When interpreting and analyzing the counts and measurements carried out on the electron micrographs, several assumptions had to be made. A crucial one is that the smooth muscle cells of the tissue examined are a uniform population of cells in terms

of cell size and extent of nexal connections. With the exception of the single cell layer of small and dark muscle cells near the submucosa (20, 25) (which is not included in the present study), the morphology of the muscle cells of this tissue in conventional transmission electron microscopy and the morphometric measurements suggest a uniform cell population. As regards the distribution of nexuses, we assumed that all muscle cells of the circular muscle coat of the guinea-pig ileum were equally supplied with these junctions; we have no evidence against this assumption (large areas devoid of nexuses can be interpreted as parts of the cell surface facing an intramuscular septum), but we cannot exclude that some muscle cells may have a richer supply of nexuses and some may have none.

A second assumption is that all clusters of intramembrane particles or pits correspond to a nexus or gap junction. That clusters of particles or pits correspond to nexuses is clearly indicated by many previous papers (see reviews in references 13, 14, 19, 22) and was confirmed in the present experiments when a plane of fracture jumped from one muscle cell to another just at the level of clusters of particles and pits. Many clusters of pits or particles, however, are exposed on one membrane face only; these we invariably assumed to be nexuses also. Since we have not prepared complementary replicas, we cannot say whether each cluster of particles on the P face of one membrane corresponds to an exactly complementary cluster of pits on the E face of the same membrane. Several studies have indicated that this is the case (e.g., references 3, 4), and this is supported by the observation that the size histogram for the nexuses we observed on P faces is virtually identical to that of the nexuses seen on E faces.

We also assumed that all the nexuses observed occurred between two smooth muscle cells. Nexuses between a smooth muscle cell and an interstitial cell are known to occur frequently in the avian gut (17). Heterologous nexuses of this type have been clearly illustrated in the intestine of the cat (26), but their number is very small and they have been seen very rarely in the taenia coli of the guinea-pig (10). In the present work, we never observed nexuses on clearly identified interstitial cells.

The quantitative results that were obtained by averaging the whole muscle cell population are summarized in Table II. The number of nexuses that we calculated to be present over the entire



surface of one muscle cell is considerable (244) but these nexuses are so small in size that altogether they constitute less than a quarter of 1% (0.212%) of the cell surface. Other values reported in the literature are: 0.75% of the cell surface examined (33 nexuses) of the guinea-pig sphincter pupillae (8), a smooth muscle which is among the richest in nexuses (9), and 0.05% of the muscle cell surface in the dog tracheal muscle (18). Much higher values have been measured in epithelial cells, e.g., in hepatocytes (1.5% of the cell surface) (15). In cardiac muscle cells of the dog, Spira (24) has calculated that 6% of the cell membrane at an intercalated disk forms nexus junctions. Matter (21) has calculated that the nexuses present in one intercalated disk between two cardiac cells of the rat contain a total of $6.7 \cdot 10^5$ particles found over one smooth muscle cell in the present experiments ($7.7 \cdot 10^4$).

The percentage of cell surface occupied by nexuses was rather similar in the three animals examined. It is known, however, that the number and the size of nexuses in smooth muscles may vary with the physiological and experimental conditions (e.g., nexuses in the rat myometrium are found only around the time of parturition [11], and nexuses increase in size and number in hypertrophied intestinal muscle cells [our unpublished results]) and also in muscles treated *in vitro* with drugs (18). Different values are obtained from other parts of the alimentary tract, and, within the small intestine, nexuses are larger and more numerous in the proximal (oral) than in the distal (aboral) portions (our unpublished results). A remarkable difference in the distribution of nexuses was observed in the longitudinal musculature of the small intestine: at the same time that we prepared the montages of the circular musculature, we prepared montages of similar extent of the longitudinal musculature and there we never ob-

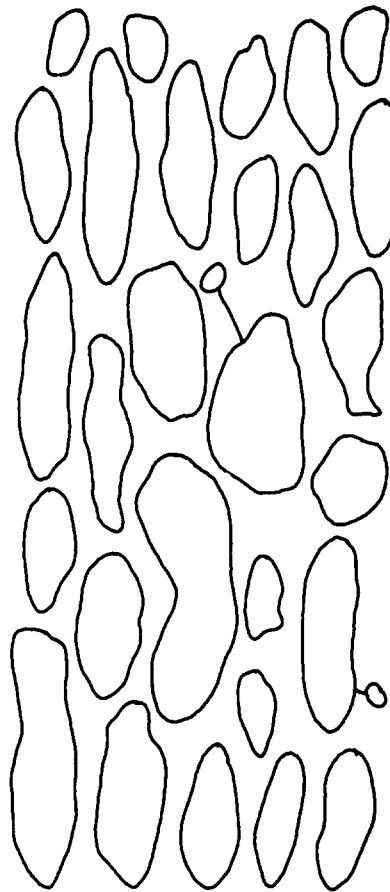


FIGURE 7 Drawings of the profiles of 28 nexuses from one montage to show their relative sizes and shapes. Two satellite nexuses are connected by a line to their respective parent nexus. The longitudinal axis of the cell runs parallel to the long side of the illustration. $\times 64,000$.

served any nexus. Although it may be premature to compare different smooth muscles from this point of view, it should be stressed that there are

FIGURE 3 Two oval-shaped nexuses exposed on the P-face of a muscle cell. They both appear to rise slightly over the general level of the cell surface, as one would expect from their appearance in sectioned material (cf. Fig. 1). Bar, $1 \mu\text{m}$. $\times 20,000$.

FIGURE 4 A nexus exposed on the P face of a muscle cell, close to a row of caveolae. Note that particle-free areas are present among the groups of tightly packed particles. Bar, $0.2 \mu\text{m}$. $\times 103,000$.

FIGURE 5 A relatively large nexus exposed on the E face of a muscle cell, accompanied by a satellite (bottom right) (see text). Bar, $0.1 \mu\text{m}$. $\times 124,000$.

FIGURE 6 An ovoid-shaped nexus exposed on the E face of a muscle cell. The lump of membrane overlying the nexus is interpreted as the P-face of a part of a cisterna of sarcoplasmic reticulum. Bar, $0.2 \mu\text{m}$. $\times 60,000$.

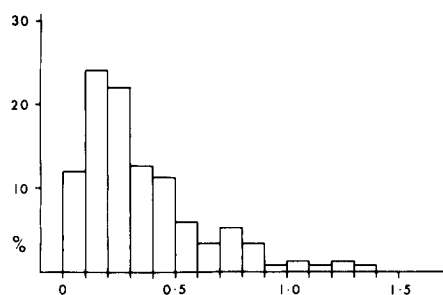


FIGURE 8 Histogram of the nexus sizes of 151 nexuses in one montage. Ordinate area in μm^2 , abscissa percentage.

large differences between various smooth muscles as regards their supply of nexuses. This point is clearly borne out by the striking difference between circular and longitudinal muscles of the small intestine, a result that confirms some previous results obtained with conventional electron microscopy (see reference 16).

It is possible, in our opinion, that—in addition to the gap junction—there are in smooth muscles other morphological correlates of electrical coupling. These should provide the coupling in muscles, such as the longitudinal layer of the guinea-pig ileum, that are devoid of nexuses. It will be

TABLE I
Quantitative Data on the Nexuses of the Circular Muscle of the Ileum

Animal (No. of montages in brackets)	Total area examined in μm^2	Total No. of nexuses counted	Σ of areas of all nexuses in μm^2	Percentage of cell surface occupied by nexuses	No. of nexuses per 1,000 μm^2
1 (7)	2,961	151	5,410	0.183	51
2 (14)	2,657	142	6,963	0.262	53
3 (2)	2,985	119	5,888	0.197	40
	8,603	412	18,261	0.212	48

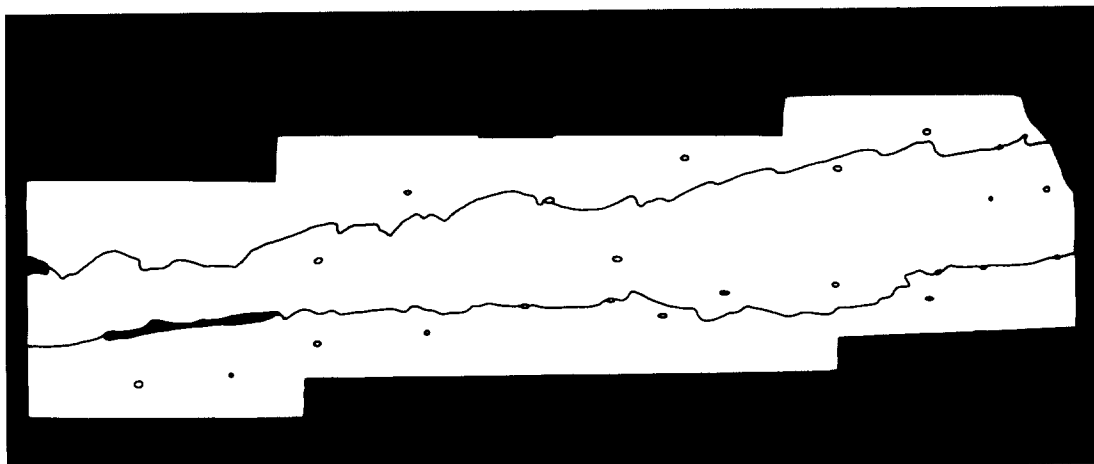


FIGURE 9 Drawing of a montage showing part of the surface membrane of three muscle cells (light areas) displaying a number of nexuses (23 small circular or oval profiles). For sake of clarity the nexuses are drawn slightly bigger than their actual size. $\times 825$.

TABLE II
Summary of the Quantitative Results

cell area:	5,074 μm^2
cell volume:	3,260 μm^3
No. of nexuses per 1,000 μm^2 :	48
No. of nexuses per cell:	244
% of cell surface occupied by nexuses:	0.212
packing density of particles or pits:	7,196/ μm^2
No. of particles in all the nexuses of one cell:	77,000

interesting to find out whether the 244 nexuses per cell (and the 77,000 presumable intercellular channels) are adequate to support the electrical coupling between the muscle cells of the circular musculature of the guinea-pig ileum, or whether additional coupling mechanisms are at work.

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