A STUDY OF NEXUSES IN VISCERAL SMOOTH MUSCLE

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ABSTRACT

Nexuses are described between the smooth muscle cells of the gizzard of the chick and the pigeon, the vas deferens of the mouse and the guinea pig, and the taenia coli of the guinea pig. The nexuses in the gizzard were examined after osmium tetroxide and potassium permanganate had been used as fixatives. Although differences in the dimensions of the unit membranes and the nexuses were noted, the results with the two fixation techniques were complementary. The distribution of nexuses within the smooth muscle tissues examined was uneven. Nexuses were still present in both small and large pieces of tissue incubated in hypertonic solutions at varying temperatures. Other experiments showed that the degree of contraction at the time of fixation did not affect the presence of nexuses in the tissue. These results indicate that nexuses between smooth muscle cells are stable under a variety of conditions.

INTRODUCTION

The occurrence of regions of fusion between apposing membranes of smooth muscle cells, termed nexuses (10), is now well documented (3, 4, 7, 10–13, 21, 23). Present evidence suggests that there are relatively low-resistance pathways between adjacent smooth muscle cells through which intercellular electrotonic coupling may occur (1, 3–5, 7, 16, 18, 24, 25). It has been suggested that the nexus and the site of electrotonic coupling are one and the same thing (2–4, 6, 7, 11, 12, 14, 24). If this is so, there is an apparent conflict in the literature between those studies which show disruption of nexuses between smooth muscle cells after incubation in hypertonic solutions (3, 12) and those studies which show that the electrotonic coupling between smooth muscle cells is unaffected by similar treatment (1, 24, 26).

This study concerns the fine structural relationships between smooth muscle cells in several tissues. Muscle cells from two preparations (taenia coli of the guinea pig and the gizzard of the chick) were exposed to a number of different prefixation treatments in order to study the effect of stress on nexuses.

MATERIALS AND METHODS

Smooth muscle preparations from the gizzard of the pigeon and the chick, the vas deferens of the mouse and the guinea pig, and the taenia coli of the guinea pig were examined. Immediately after the dissection of the animal, cold fixative was applied to the organ under study, and a convenient small piece was excised. The tissue that was removed was immediately placed in buffered fixative and left for between 15 and 30 min at 4°C, after which it was dissected into pieces about 1 mm^2 and fixed for a further period at the same temperature. Two fixation techniques were used, both fixatives being buffered with Veronal acetate at a pH of 7.3. In the first method, a 1% solution of osmium tetroxide in buffer was used for a total fixation time of 1 hr. The other technique involved the use of a 1% solution of potassium permanganate in buffer for a total fixation time of 2 hr. After fixation the tissue was rinsed in 20% acetone

and dehydrated, the whole procedure taking not more than 30 min. After a period of 4 hr in a 50% mixture of Araldite and acetone, the tissue was finally embedded in Araldite by using a vacuum embedding chamber.

Small blocks of the material embedded in Araldite were trimmed, and sections were cut on a Huxley ultramicrotome. The sections were placed on uncoated 300-mesh copper grids. Material that had been fixed in potassium permanganate was examined unstained. Tissue fixed with osmium tetroxide was stained in a solution of lead citrate (prepared by adding 0.5 ml of 10 N NaOH to 0.04 g of lead citrate in 10 ml of distilled water) for 2 min, followed by staining in a 2% solution of uranyl acetate for 3 min. Sections were examined in an Hitachi HU-11b electron microscope at 50 kv.

Tissue from the gizzard of the chick and the taenia coli of the guinea pig was fixed in potassium permanganate under the following conditions.

Fixation of Material in Different States of Contraction

Experiments were performed on tissue from the taenia coli of the guinea pig to determine what tension changes occurred during fixation. Lengths of taenia coli were suspended in a 10 ml organ bath; one end of the tissue was clamped, and the other end was attached to the sensing arm of a Nihon-Kohden force-displacement transducer. The transducer was arranged to record isometric tension changes and was coupled to a pen recorder. While being bathed in modified Krebs' solution (for taenia coli, see reference 9), the tissue was stretched until it was just taut. The Krebs' solution was then replaced by fixative. There was a marked increase in tension. After 2–3 min the tension decreased towards its initial value. If prior to the application of the fixative the tissue was tensed to the maximal value recorded during fixation of a similar piece of tissue, then when the fixative was applied there was little change in tension recorded throughout the fixation period. Thus the tissue was presumably at maximum tension for this time.

In the light of these experiments, tissue was fixed either (*a*) unrestrained, (*b*) stretched until it was just taut, or (*c*) stretched as far as possible without breaking.

Fixation of Material after Different Periods of Incubation

Tissue was incubated in modified Krebs' solution (for taenia coli, see reference 9; for gizzard, see reference 15) at room temperature and at 38°C for periods from 1 to 6 hr prior to fixation.

Fixation of Material after Incubation in Hypertonic Solution

Pieces of tissue from 100 μ in diameter by 2 mm long up to 3 mm in diameter by 5 mm long were incubated in solutions of different tonicities for 1 and 2 hr at both 21°C and 38°C. The hypertonic solutions were prepared by addition of the required amount of sucrose to the normal Krebs' solution for the taenia coli and the gizzard. The tonicities used for the taenia were 330 (normal), 480, 640, and 960 mM, and for the gizzard they were 382 (normal) 573, 764, and 1146 mM.

Measurement of Micrographs

Positive transparencies of the relevant part of micrographs were made at a magnification of 7. The slides were then projected to enable measurements to be made at a final magnification of approximately 20 million times. Mean values were obtained from a large number of readings. Yamamoto (27)

TABLE I

Measurements of Unit Membrane and Nexus

Type of structure	No. of micrographs	Inner leaf	Intermediate leaf	Outer leaf	Total
Unit membrane (OsO_4 fixed)	21*	23	27	23	73
Unit membrane (permanganate fixed)	29	21	25	21	67

		Inner leaf	Inter-mediate leaf	Fused outer leaf	Inter-mediate leaf	Inner leaf	
Nexus (OsO_4 fixed)	18	41	27	27	27	41	163
Nexus (permanganate fixed)	31	21	26	28	26	21	122

* 10 measurements were made from each micrograph by using projected slides. The values given in the above chart are the mean values, measured in Ångstroms.

has made it clear that, even when all procedures are carried out under identical conditions, the results are subject to a number of errors, including estimation of focus, difficulty in identifying edges, and variation in photographic processing. Yamamoto (27) further points out that even the use of a densitometer does not fully resolve the uncertainty.

However, in this present study, the mean values that were obtained are only used to indicate the marked differences in some of the component layers of the unit membrane and the nexus.

RESULTS

Fine Structure of the Nexus after Different Fixation Procedures

A comparison of the structure of nexuses in smooth muscle after fixation with osmium tetroxide and potassium permanganate was made on tissue from the gizzards of the chick and the pigeon.

Table I is a chart of the measurements of the components of the unit membrane and nexus between adjacent smooth muscle cells from the gizzard after osmium tetroxide and potassium permanganate fixation. After osmium tetroxide fixation, the unit membrane was a triple-layered structure measuring 73 A across on average, and the nexus was a quintuple-layered structure measuring 163 A on average. At the nexus the inner leaves of the unit membrane were thickened; the outer leaves fused to form a single electron-opaque layer little wider than the thickness of a single leaf. The essential structure of the unit membrane and nexus fixed with potassium permanganate was similar to that of the unit

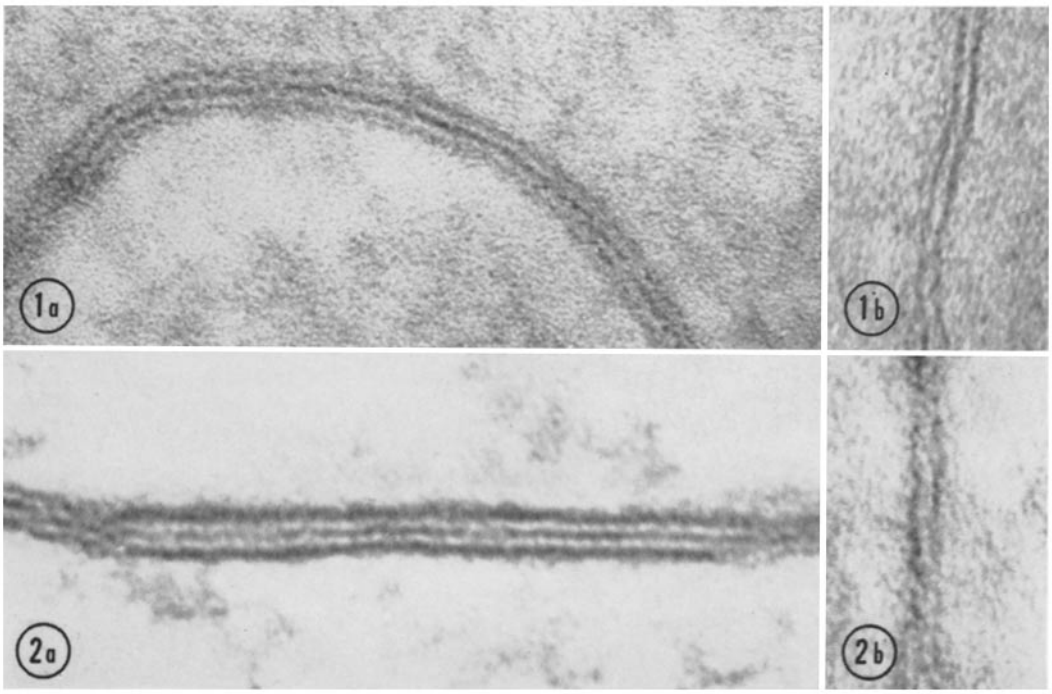

FIGURE 1*a* Nexus between two muscle cells in the gizzard of the chick, showing the quintuple-layered structure after fixation with potassium permanganate. × 500,000.

FIGURE 1*b* Unit membrane of a smooth muscle cell of the gizzard of the chick fixed with permanganate. The cytoplasmic side of the unit membrane is to the left of the micrograph. × 460,000.

FIGURE 2*a* Nexus in the gizzard of the chick after fixation with osmium tetroxide. The structure illustrated should be compared to that in Fig. 1*a*. × 380,000.

FIGURE 2*b* Unit membrane of the smooth muscle of the gizzard of the chick demonstrated with osmium tetroxide fixation. The cytoplasmic side of the unit membrane is to the left of the micrograph. This figure should be compared to Fig. 1*b*. × 500,000.

membrane and nexus fixed with osmium tetroxide. The unit membrane measured 67 A across on average, and the nexus 122 A on average. The outer leaves of the unit membrane fused at the nexus to give a single electron-opaque layer little more than half their combined width. Figs. 1 and 2 show the structure of the unit membrane and the nexus after the two fixation procedures.

After osmium tetroxide fixation, the triple-layered nature of the unit membrane was often not resolvable, the structure appearing as a single dense line. Potassium permanganate fixation, however, preserved the details of the unit membrane and the nexuses very clearly.

Types and Distribution of Nexuses

The muscles of the gizzard of chick and pigeon, the vas deferens and taenia coli of guinea pig, and the vas deferens of the mouse were all examined. The morphological features of the nexuses were not found to differ markedly in any way from one tissue to another. There appeared to be three main types of nexal region between muscle cells, although it was not clear whether these types were simply different variations of the same feature.

A more extensive study of the nexus was made on the gizzards of the chick and pigeon after both potassium permanganate and osmium tetroxide fixation. The first type of nexus consisted of small plaquelike junctions with little or no protrusion. Figs. 3 and 4 show the same morphological type of nexus with both types of fixation. Fig. 5 shows a nexus formed between two muscle cells with no protrusion of one cell into another. The length of these small regions of fusion of the outer leaves of the cell membranes was rarely more than 0.5 μ.

The second form of nexus was characterized by a protrusion of one cell into another, usually over a distance of about 2 μ (Fig. 6). The protrusion had a narrow neck, in most cases, with a terminal bulb of larger diameter. The nexus in such a protrusion was nearly always confined to the terminal bulb. The nexuses formed by these protrusions were demonstrated with both fixatives used; Figs. 7 and 8 are transverse sections through the region of the terminal bulb. Fig. 9 shows a transverse section

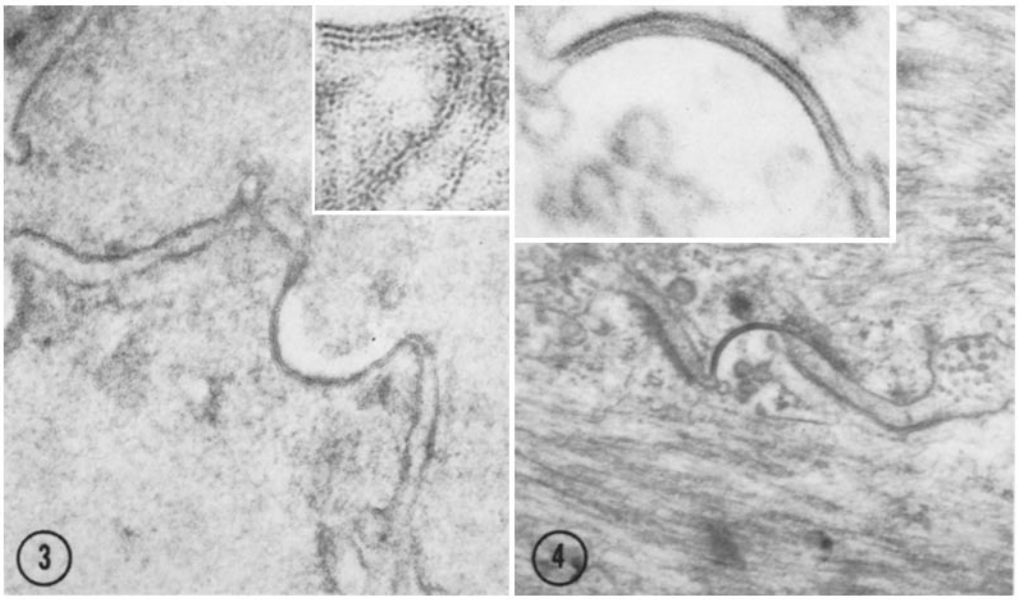

FIGURE 3 Small plaquelike nexus between two muscle cells in the gizzard of the chick demonstrated after permanganate fixation. There is little interdigitation of the muscle cells. × 80,000. Inset, enlargement of part of nexus. × 220,000.

FIGURE 4 Nexus, similar to that in Fig. 3, from the gizzard of the chick fixed in this case with osmium tetroxide. The same range of size and gross morphological form of nexus is demonstrated after both fixation procedures. × 45,000. Inset, × 155,000.

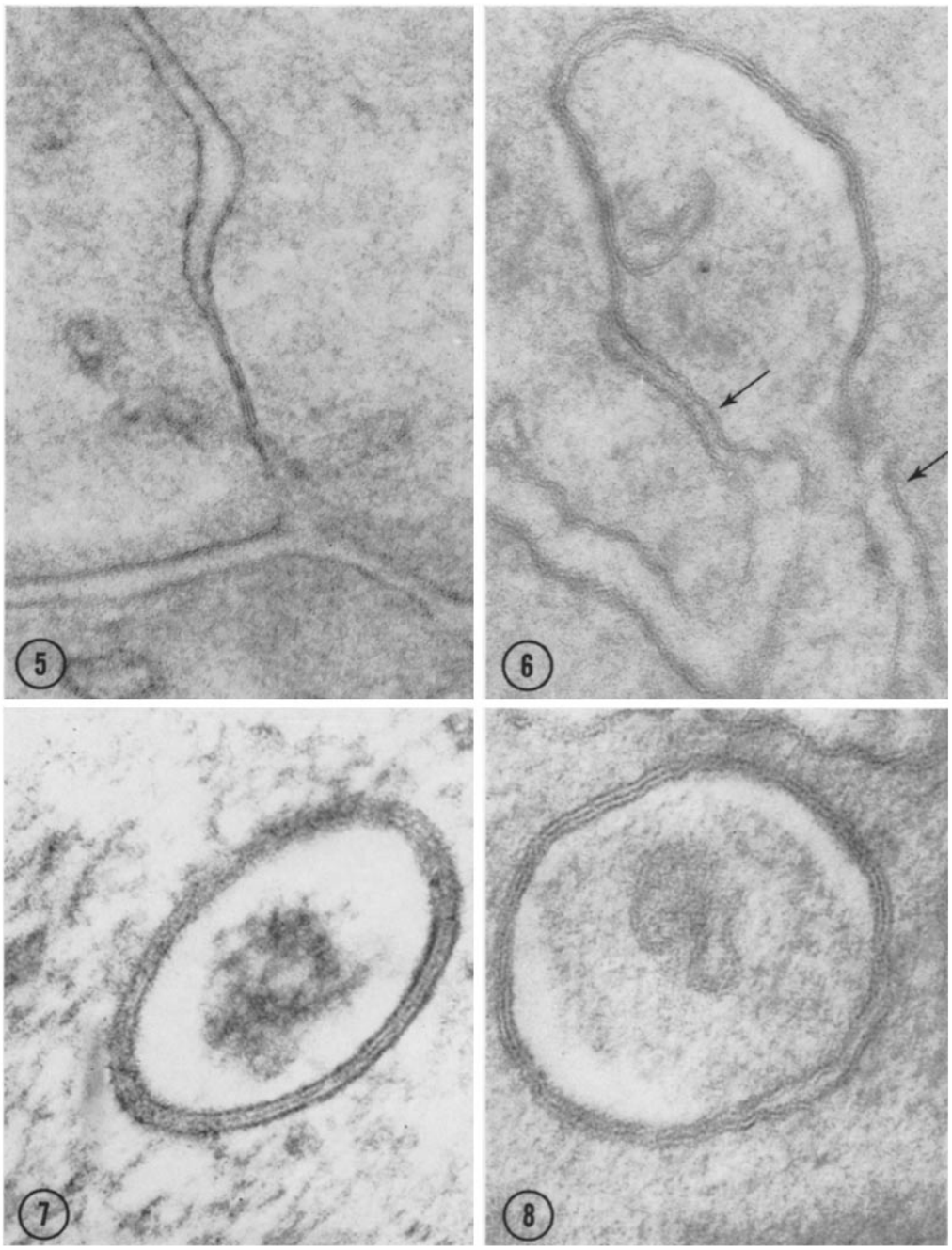

FIGURE 5 Small plaquelike nexus in the vas deferens of the guinea pig, showing no interdigitation of the muscle cells. Permanganate fixation. × 125,000.

FIGURE 6 Small protrusion of one smooth muscle cell into another, showing nexus formed in the enlarged bulb but not at the narrower neck region (arrows). Chick gizzard fixed with permanganate. × 150,000.

FIGURE 7 Transverse section of protrusion between smooth muscle cells in the gizzard of the chick, showing a nexus. Osmium tetroxide fixation. × 175,000.

FIGURE 8 A type of nexus similar to that in Fig. 7, demonstrated in the gizzard of the chick after permanganate fixation. This study has shown that the same gross morphological types of nexus are present with both fixatives. × 210,000.

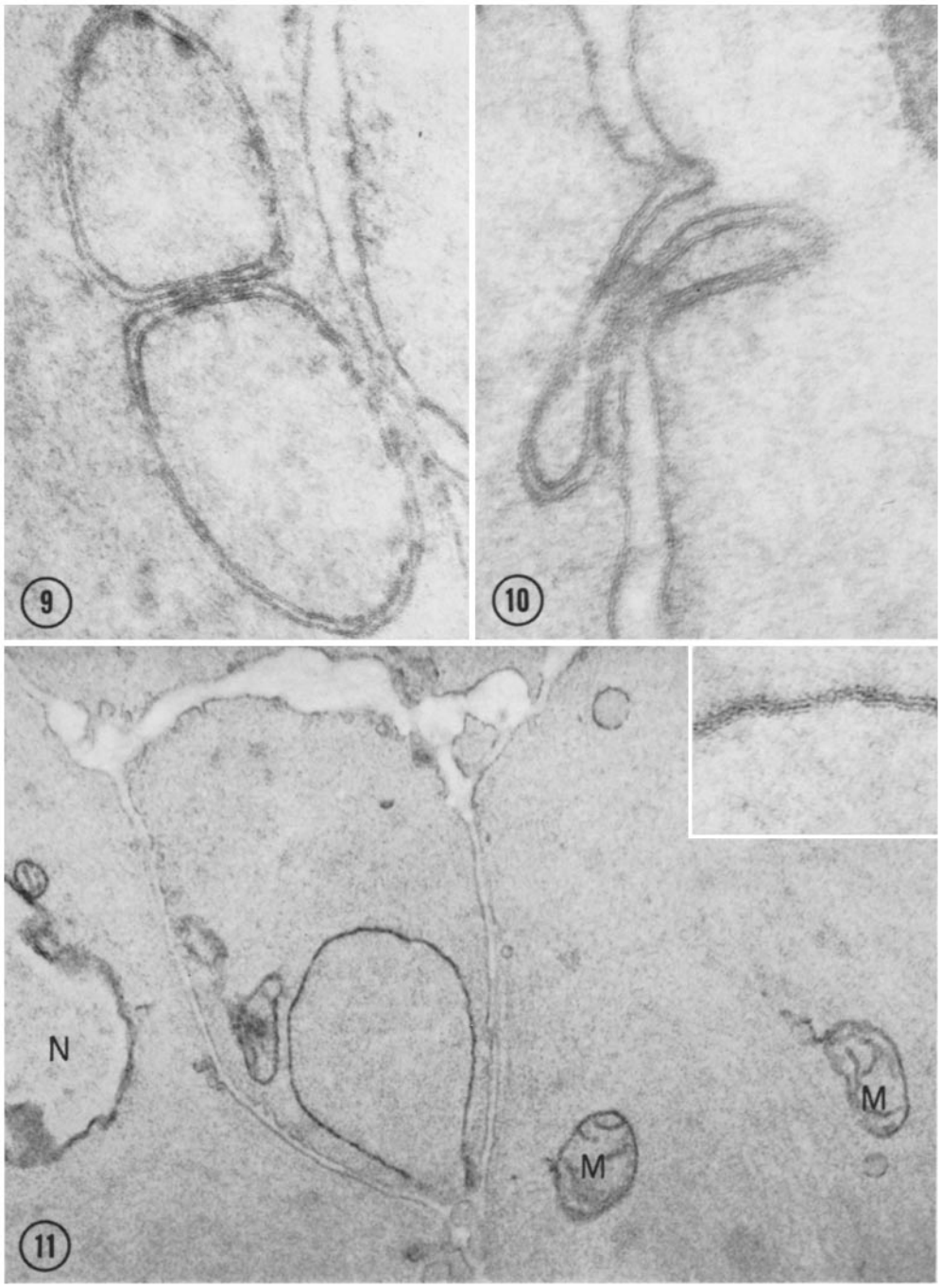

FIGURE 9 Transverse section of two protrusions into a muscle cell of the chick gizzard. This section appears to pass through the narrow neck regions of the protrusions where nexuses are not formed. Serial sections have shown that such protrusions form nexuses in a deeper plane of section. Permanganate fixation. × 90,000.

FIGURE 10 Protrusions between some cells are reciprocal and often very small. Serial sections usually show areas where the membranes are fused in even the smallest protrusions. Permanganate fixation of gizzard of the pigeon. × 130,000.

FIGURE 11 Transverse section through the circular muscle layer of the vas deferens of the guinea pig, showing large protrusion between muscle cells. These protrusions can be up to 5 μ in diameter and 10–12 μ in length. The nucleus of one muscle cell is shown (*N*), as well as mitochondria (*M*). Permanganate fixation. × 40,000. Inset, × 150,000.

through the neck region of two protrusions into the same muscle cell; serial sections through such protrusions demonstrated the nexus formed at the terminal bulb. On some occasions the protrusions between cells were reciprocal (Fig. 10). Examination of material at high magnifications sometimes revealed protrusions with the neck region less than 500 A in diameter.

The third type of nexus is shown in Fig. 11. It consisted of a very large protrusion of one cell into another, sometimes up to 5 μ in diameter and 10 μ in length; nexuses could be demonstrated in serial sections over large areas of the contact surface between the two cells involved.

The distribution of nexuses within the types of smooth muscle tissues examined was difficult to determine. It was clear from an examination of thin sections cut from any of the muscles studied that the distribution of nexuses was not even. The highest count made was in a longitudinal section of chick gizzard fixed with osmium tetroxide where 21 protrusions occurred between five muscle cells over 15 μ of their length, while in other parts of the same section there were no nexuses.

Effects of Varying Prefixation Treatment

In order to assess the stability of the nexus, a number of variations were made in the preparation of the tissue for examination. It was apparent, however, since the distribution of nexuses in any material was not even, that only a qualitative assessment of the effects of the procedures could be made.

Incubation of smooth muscle from the chick gizzard and the taenia coli of the guinea pig for periods up to 6 hr in Krebs' solution, at either 38°C or room temperature, did not obviously increase the numbers of nexuses observed. Similarly, nexuses were found whether the material was fixed *in situ* and then dissected and fixed further or was dissected and chopped up before fixation. Tissue taken from the longitudinal muscle layer of the intermediate muscles of the gizzard (see reference 20) was always highly contracted after fixation. This material was typified by a considerable distortion of the profiles of both the individual muscle cell and its nucleus. The even distribution of the myofilaments in such tissue was disarranged; areas of almost clear cytoplasm alternated with areas congested with myofilaments. Tissue from other parts of the gizzard and the taenia coli that was fixed unrestricted generally showed smoother profiles of the muscle cells and the nuclei and an even distribution of the myofilaments and was thus clearly not in a maximally contracted state. The intercellular space between the muscle cells in this tissue was of approximately constant width in a transverse section of a muscle bundle. The tissue fixed at approximately resting length and that fixed stretched taut showed slightly greater distortion of the profiles of the muscle cells and the intercellular space. However, in none of this tissue were there any obvious differences in the number of nexuses observed. Finally, pieces of tissue from about 100 μ by 2 mm to 3 mm by 5 mm in size were incubated for both 1 and 2 hr at 21° and 38°C in modified Krebs' solutions of different tonicities. When these tissues were examined it was clear that the muscle cells had undergone much shrinkage, especially at the high tonicities, and that this shrinkage had caused a distortion of their normal appearance and arrangement. Fig. 12 shows a small protrusion between two muscle cells from material which had been incubated in hypertonic solution; the arrangement of the membranes of the muscle cells is distorted, but the nexus formed in the bulb of the protrusion is intact. Fig. 13 shows a larger protrusion with the muscle cells separated but with the nexus still clearly identifiable. Fig. 14 shows a very large protrusion between two muscle cells; although the protruding cell is considerably distorted due to osmotic shrinkage, the large area of nexus remains completely intact. There was no apparent difference in the over-all numbers or appearances between nexuses seen in large pieces of tissue (3 mm by 5 mm) incubated in hypertonic solution and those seen in small pieces (100 μ by 2 mm) incubated in the same solution. There was no difference in the nexuses after incubation for 1 or 2 hr in hypertonic solutions prior to fixation. Strongly contracted material often showed distortion of the protrusions similar to the distortion seen in tissue incubated in hypertonic solution, but in neither case was any rupture of nexuses seen. Indeed, those areas of membrane fusion appeared to have considerable resistance to rupture.

DISCUSSION

It is clear from previous work (3, 4, 10, 11, 12, 21, 23) and the present results that potassium permanganate is a more suitable fixative than is osmium tetroxide when one is examining nexuses

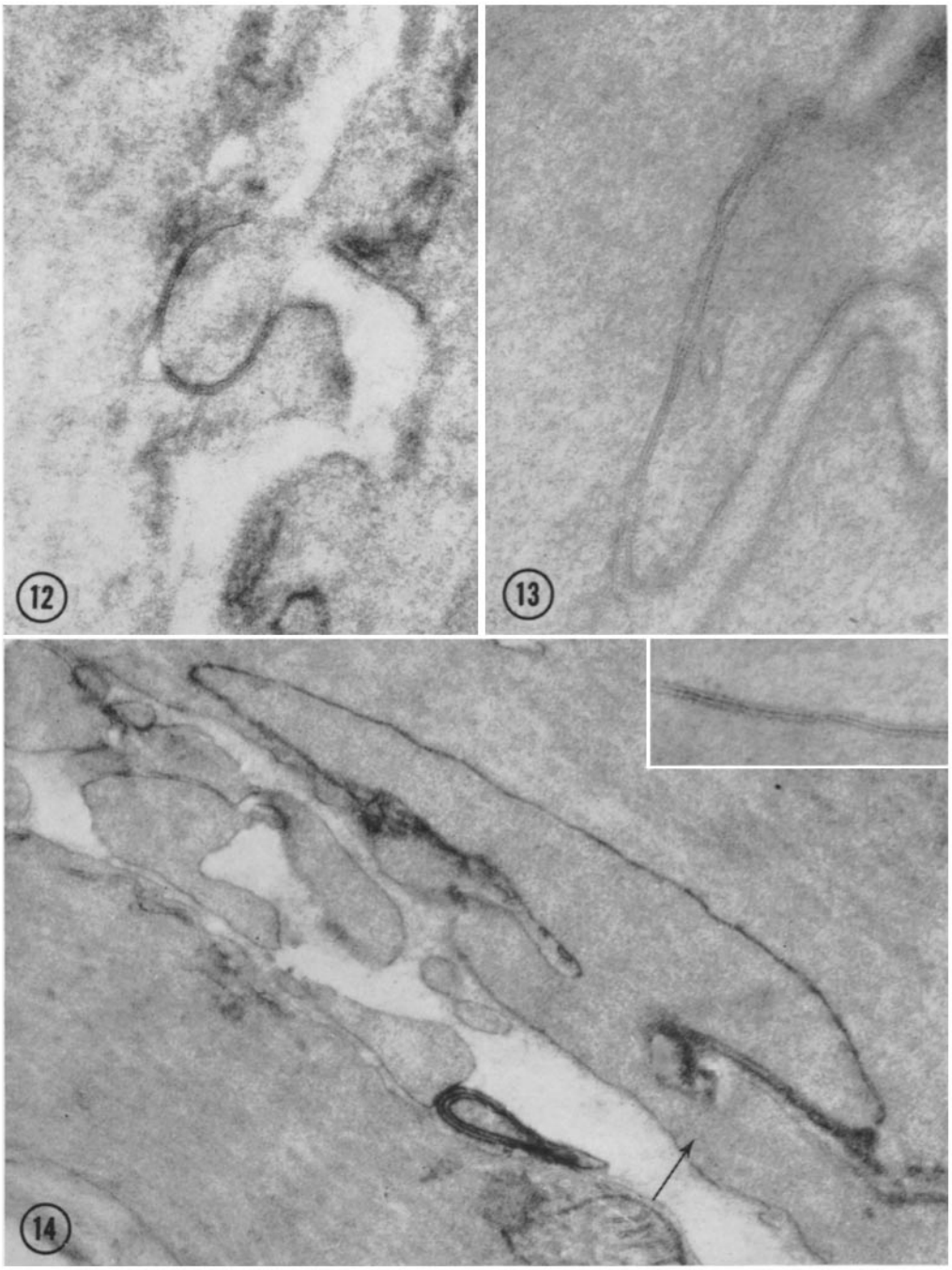

FIGURE 12 Small protrusion between two muscle cells in the taenia coli of the guinea pig, showing a nexus. The tissue is much distorted due to incubation for 2 hr in Krebs' solution made hypertonic (960 mM) with sucrose prior to permanganate fixation. × 100,000.

FIGURE 13 Nexus between two cells of the taenia coli of the guinea pig. Shrinkage of cells incubated in hypertonic Krebs' solution (620 mM) causes separation of the muscle cells and distortion of the typical shape of protrusions between cells but no rupture of the nexus. × 100,000.

FIGURE 14 A large protrusion between adjacent muscle cells of the taenia coli of the guinea pig, unaffected by incubation for 2 hr in hypertonic ringer (960 mM). The nexus (see inset) formed by the protrusion shown here is normal, but the remainder of the protruding cell (arrow) is considerably distorted. × 33,000. Inset, × 160,000.

in smooth muscle tissues, since the nexal structure is more clearly preserved by permanganate fixation. A number of workers have demonstrated nexuses between smooth muscle cells after osmium tetroxide fixation (13, 17, 19, 21, 22). In those cases where the details of the nexuses are clearly shown (13), these structures are seen to be comparable to some of the structures seen in the present study. Furthermore, the present study has shown that, in the gizzard of the chick and of the pigeon, similar numbers, ranges of size, and types of nexuses are readily discernible with both fixatives.

In thin sections of material fixed in permanganate, the triple-layered structure of the unit membrane is clearly identified throughout most of the cell profiles. After fixation with osmium tetroxide similar preservation of the unit membrane is infrequent. In material fixed with osmium tetroxide, the fused outer leaves of the unit membranes at the nexus appear narrower and, in places, less electron opaque than do the inner leaves. The width of the nexus is more than twice the width of a single unit membrane. In material fixed with permanganate, the nexus is less than twice the width of a single unit membrane, which is mainly due to the outer leaves of the adjacent unit membranes fusing to form a layer less than one-half their combined width.

The distribution of nexuses within the smooth muscles examined in this study has been shown to be irregular. Some areas examined showed many nexuses, often between the same two muscle cells. In other regions, few, if any, nexuses were apparent.

Physiological studies on the taenia coli (7, 18, 24) and the vas deferens (5, 25) of the guinea pig, on the vas deferens of the mouse (16), and on the gizzard of the chick (Bennett, T. Data in preparation) indicate that there is electrical coupling between most cells in these smooth muscle tissues. Since nexuses are assumed to be the sites of electrical coupling, they should occur between most smooth muscle cells. The uneven distribution of the nexuses, therefore, suggests that the muscle cells may be arranged in such a way that the nexuses occur together in the same general areas and are not distributed evenly throughout the muscle bundles.

There is great variation in the extent of nexuses between smooth muscle cells, ranging from very small interdigitations no more than 500 A in diameter to large protrusions of one cell into another. In these large protrusions, the fused membranes can be followed continuously for 10–12 μ in a single thin section.

Lane and Rhodin (19) studied the relationships between smooth muscle cells in the mouse vas deferens and intestine. They claimed that, in the vas deferens, cell contacts were of the peg and socket type with no obvious nexal fusion of the adjacent membranes. They stated that cell contacts in the intestine were planar with nexal fusion of the contacting cell membranes. Lane and Rhodin suggested that such anatomical differences might be correlated with the electrophysiological characteristics of these two tissues. They assumed that no electrotonic coupling occurred between the smooth muscle cells of the mouse vas deferens, and they explained this lack of coupling on the basis of the observed absence of nexuses from this tissue.

It has since been shown that electrotonic coupling does occur between the smooth muscle cells of the mouse vas deferens (16), and the present study has shown that a normal range of nexal types is found in this tissue.

It is at present not clear whether the different forms of nexuses seen in the tissues examined are merely variations of the same type of structure or if the differences in size and form have physiological significance.

Previous reports (3, 11, 12) have described the disruption of nexuses in smooth muscle tissues after treatment similar to that used in the present study. Barr et al. (3) and Dewey and Barr (12) have claimed that treatment of the taenia coli of the guinea pig with hypertonic solutions disrupts the nexuses between the smooth muscle cells and thereby blocks the propagation of action potentials. Tomita (24) has shown that in hypertonic solutions the electrical properties of the smooth muscle cells of the taenia coli were indistinguishable from normal. Barr et al. (3) suggest that Tomita's results (see reference 24) might be due to his using the whole taenia coli rather than smaller pieces of tissue as they did. The smallest pieces of tissue used by Barr et al. were 200 μ in diameter. Tomita (24) used muscle bundles as narrow as 150 μ and still could detect no abnormality in the electrical responses of the cells. Thus the objection raised by Barr et al. (3) would seem to be unfounded.

Since the present study has shown that under all experimental conditions employed the nexuses between the smooth muscle cells showed no signs of disruption, Tomita's results are consistent with the assumption that the nexus is the site of electrical coupling between smooth muscle cells.

In order to demonstrate block of action potential propagation when the taenia coli was exposed to hypertonic solutions, Barr et al. (3) immersed strips of the tissue in hypertonic solutions and recorded electrical activity from different points along its length by using external electrodes. When bathed in hypertonic solution, the taenia coli loses its spontaneity (24); this has been shown to be due to a hyperpolarization of the membrane resulting from an increase in intracellular potassium (8). Such a hyperpolarization may well explain the observations of Barr et al. (3), especially since it has been shown that increasing the external potassium concentration restores the normal activity of the taenia coli bathed in hypertonic solution (24). If hypertonic solutions did disrupt the nexuses, it is difficult to see how normal activity could be restored by an increased extracellular potassium concentration.

Thus, while the nexus seems likely to be the site of electrotonic coupling between adjacent smooth muscle cells, there is still no proof that it is.

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