Electron Microscopic Observations on the Innervation of the Smooth Muscle

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Intestinal smooth muscles (the rat and guinea pig ileum, guinea pig taenia coli and rabbit colon), and urogenital smooth muscles (vas deferens, seminal vesicle, urinary bladder and ureter of the rat and guinea pig) were observed with the electron microscope to study the innervation of the autonomic nerve in the smooth muscle tissue.

In the intestinal smooth muscles, the nerve endings occurred as a large aggregate, but no single axon was present. In the urogenital smooth muscles, on the other hand, an abundant distribution of the single axons was observed among the smooth muscle fibers.

The synaptic vesicles found in the axons of the intestinal smooth muscles were mainly agranular, whereas those of the urogenital smooth muscles were granular vesicles partly mixed with agranular ones. In the smooth muscle layer of the rabbit colon, the nerve endings contained characteristically large granular vesicles that could not be observed usually in other smooth muscle tissues.

Since Burnstock and Holman\textsuperscript{1} reported the excitatory junction potentials in the smooth muscle cells of the guinea pig vas deferens, morphological features of the autonomic nerve endings in the smooth muscle layers have been studied circumstantially in the vas deferens preparations of the rat\textsuperscript{2} and guinea pig.\textsuperscript{3}

Recently, in the smooth muscle of the rabbit colon\textsuperscript{4} and in the guinea pig taenia coli,\textsuperscript{5} electrophysiological studies have revealed the presence of the inhibitory potentials, which are considered to be evoked, at least partly, by the inhibitory adrenergic nerve fibers in the tissue.

It will be of interest to study the ultrastructure of the nerve endings of the intestinal smooth muscle to compare with those of the vas deferens where the main innervation is thought to be adrenergic, and further to identify two different types of nerve endings which would represent cholinergic and adrenergic nerve fibers, respectively.

We have observed many smooth muscle preparations of rats, guinea pigs and rabbits with the electron microscope and found that the intestinal and the urogenital smooth muscles could be separated distinctly by the difference in the distribution of the terminal single axons in the smooth muscle layer. The termi-
nal single axons were not present in the intestinal smooth muscles, whereas in the urogenital smooth muscles, terminal single axons were found very abundantly.

Two types of synaptic vesicles, agranular and granular, were found in the terminal axons. Agranular vesicles were the main component of the axons of the intestinal smooth muscles, and the granular vesicles were main component of the axons of the vas deferens. However, it was not possible to identify exactly the axons with adrenergic or cholinergic nerve terminals according to the content of vesicles.

MATERIALS AND METHODS

The present observations were carried out on the intestinal smooth muscle preparations from rats' and guinea pigs' ileum, guinea pigs' taenia coli and rabbits' colon, and on the urogenital smooth muscles from rats' and guinea pigs' vas deferens, seminal vesicle, urinary bladder and ureter.

The specimens were quickly cut out from the animal and immersed in ice cold fixatives with moderate tension applied to the muscle. The fixatives used were 1.2% potassium permanganate, 1% osmium tetroxide or the equivalent volume mixture of the potassium permanganate and osmium tetroxide. They were buffered with veronal acetate to pH 7.4.

After about 2 to 6 hours’ fixation in cold fixatives, the specimen blocks were dehydrated with ascending series of ethanol.

The specimens were embedded in Epon 812 according to the method of Luft. Thin sections were cut with glass knives on a Porter-Blum MT-2 microtome and were stained with 2.5% uranylacetate and lead citrate and examined with a Hitachi HS–7 electron microscope.

OBSERVATIONS

1) Nerve fibers

Large bundles of unmyelinated nerve fibers were found in the wide extracellular spaces of the smooth muscle layers (Figs. 1 and 2). At this level of nerve trunk, the distribution and the content of the nerve fibers in the intestinal and in the urogenital smooth muscles were essentially similar.

A large number of unmyelinated nerve fibers were enclosed with the cytoplasm of Schwann cell and by thin flattened cells of perineurium.

The axons contained fine neurofilaments and mitochondria, but no synaptic vesicles at this level. As nerve bundles ramified deep into the smooth muscle layer, the number of the axons in the nerve bundle decreased gradually (Figs. 4–6). And these small nerve bundles were frequently found to be accompanied with blood capillaries (Fig. 4).

In Auerbach’s plexus in the intestinal smooth muscle, several nerve cells were found being surrounded by many axons and Schwann cell cytoplasm (Fig. 3).
In this region, longitudinal and circular muscle layers were separated by a wide extracellular space containing many collagen fibrils and fibroblasts.

In some instances, a few myelinated nerve fibers were found in a large bundle of unmyelinated nerve fibers. They could be found both in the intestinal smooth muscle of the taenia coli (Fig. 7) and in the urogenital smooth muscle of the guinea pig vas deferens (Fig. 18). Their origin and function were uncertain.

2) Nerve endings in the intestinal smooth muscles

In the intestinal smooth muscles, the axons were found as a group in a relatively wide extracellular spaces which were frequently connected with larger spaces where numerous nerve bundles, fibroblasts and blood capillaries were present (Figs. 4 and 6). No single nerve ending was observed even in the narrowest extracellular spaces.

The terminal axons of the guinea pig taenia coli and of the rat ileum (Figs. 8–11) contained many synaptic vesicles and aggregated together to form a nerve bundle.

Between these nerve bundles, there were packed groups of smooth muscle cells which did not seem to be closely related to the nerve bundles. It seemed likely that, in the intestinal smooth muscles, the innervation was limited only to the smooth muscle cells which were facing the larger extracellular spaces and the rest of muscle cells were independent of the nerve supply.

Figs. 10 and 11 show the instances of the most close apposition of naked axons with intestinal smooth muscle cells. Even in these circumstances, there remained a wide space of about 1,300Å between the axon and the muscle cell.

Also in the circular muscle layer of the rabbit colon, axons occurred in a group and no single axon was present. An interesting finding in this preparation was the abundant occurrence of the pinocytotic vesicles beneath the smooth muscle cell membrane opposing the axon and Schwann cell cytoplasm (Figs. 14, 15 and 17). There were neither thickening of the muscle cell membrane nor the aggregates of the synaptic vesicles at the localized portion of the axonal membrane, though Thaemert8 reported that these changes were present in the smooth muscle of the rat intestine.

3) Nerve endings in the urogenital smooth muscles

In the urogenital smooth muscles, abundant single axons were found in the smooth muscle layers. In the vas deferens, single axons occurred either in the narrow intercellular spaces with remnant cytoplasm of Schwann cell (Fig. 20), or buried deep in the grooves of muscle cells (Figs. 21–23).

The Schwann cell cytoplasm usually disappeared at the narrow space between the buried axon and the muscle. In the vas deferens, the narrowest spacing was measured to be about 200Å.

In some instances, the aggregates of pinocytotic vesicles (Fig. 22) were
found beneath the smooth muscle cell membrane of the axonal contact. They were sometimes observed on the muscle cell membrane not closely related to the axon (Fig. 23).

The smooth muscle layer of the guinea pig seminal vesicle also contained single axons and granulated synaptic vesicles comparable to those of the vas deferens (Figs. 24 and 25), though good preservation of the fine structure was considerably difficult in this tissue.

The smooth muscle of the urinary bladder of the guinea pig and the rat also contained single axons in the intercellular spaces (Figs. 26 and 28) and in the groove of the muscle cell (Fig. 27).

In the ureter, nerve terminals in the smooth muscle layer were very sparse but as far as they could be seen they appeared as a single axon present closely attached to the muscle cell. In Fig. 29, the terminal axon was sectioned longitudinally, showing the possibility of the synapse en passage.

4) Synaptic vesicles

As is well known, peripheral autonomic nerve fibers in the smooth muscle contain two types of synaptic vesicles according to the electron density of the substance in the vesicle. They were agranular vesicles and granular or cored vesicles, and it is generally accepted that agranular vesicles are referred to the cholinergic nerve fiber and granular vesicles to the adrenergic nerve fiber.

In the intestinal smooth muscle, the synaptic vesicles were mainly agranular, but in the urogenital smooth muscle, the synaptic vesicles were predominantly granular, being in good agreement with the known physiological evidence of the autonomic nerve innervation of these tissues.

In the guinea pig taenia coli and in the rat ileum, the predominant axons contained agranular vesicles of mean diameters of 370–600Å, with occasional large faintly stained granular vesicles of 850–1,000Å in diameter (Figs. 10 and 11). These axons are supposed to be cholinergic nerve fibers.

In some instances, axons contained granular vesicles of the mean diameter of 400Å with central core of 150Å were observed in these tissues (Fig. 8). The proportion of these granular vesicle containing axon to agranular vesicle containing axon in the intestinal smooth muscle was not determined easily due to the large variation of their appearance in the section. In the rat ileum, for example, granular vesicle containing axons were found abundantly around the ganglion cells of the Auerbach's plexus (Fig. 3). These axons may be adrenergic nerve fibers in the intestinal smooth muscle, but a more detailed histochemical analysis is needed to identify them clearly.

In the rabbit colon, a large number of terminal axons contained very densely stained large synaptic vesicles of 800–1,400Å in diameter (Figs. 12–17). The size of these granular vesicles is larger than the usual granular vesicles found in the adrenergic terminals of the vas deferens preparation.
In the vas deferens, as already reported, the majority of the terminal axons contained synaptic vesicles with dense cores (Figs. 19–23). Agranular vesicle containing axons were also present in the vas deferens but their occurrence was practically insignificant.

In the guinea pig seminal vesicle and in the urinary bladder, axons were found to contain granular vesicles similar to those of the vas deferens, but their occurrence was observed to vary greatly from one preparation to another (Figs. 24–28).

DISCUSSION

Lane and Rhodin reported the difference in the distribution of the nerve endings and in the mode of intercellular connection of the smooth muscle cells of the mouse intestine and vas deferens. In the present observation, a similar difference in the distribution of the nerve endings between the intestinal and the urogenital smooth muscles is confirmed on a variety of smooth muscle tissues from various animals.

In the intestinal smooth muscles, the nerve endings aggregate to form a nerve bundle and occur in a large extracellular spaces.

It seems most likely that nerve fibers innervate only the outermost smooth muscle cells facing the large extracellular space, and the rest of muscle cells in the bundle are not directly innervated by the nerve elements and the excitation may be conducted through muscle fiber to fiber in this bundle.

The number of smooth muscle cells composing such a bundle seems to be in a range of some 20 to 100 cells per bundle, though it could not be determined accurately.

If such innervation is commonly the case, the excitatory and the inhibitory nerve fibers should influence the same muscle fiber on relatively restricted area. And such morphological arrangement seems to be in favor of the explanation of the complex response of the smooth muscle cell showing both excitatory and inhibitory responses according to the slight alteration in the mode of electrical stimulation.

Contrary to the intestinal smooth muscles, the urogenital smooth muscles contain abundant single axons. The occurrence of pinocytotic vesicles beneath the muscle cell membrane of the axonal contact, though not frequently observed, seemed to represent the active transfer of substance around this region. And it seemed to be indicative of the presence of the synaptic transmission in the vicinity, whether the axon opposing this region is synapse en passage or the true anatomical ending.

In the circular muscle layer of the vas deferens of guinea pig, many single axons are present and sometimes two or more axons could be observed in close contact with single muscle cell. Through the observations of many sections, the authors gained the impression that every muscle cell of vas deferens and urinary
bladder is practically multi-innervated by more than one single axons. With respect to this problem, Caesar et al.\textsuperscript{10} on the mouse urinary bladder, Richardson\textsuperscript{2} on the rat vas deferens, supported the view of the multi-innervation of every muscle cell. Merrillees et al.'s\textsuperscript{3} findings on the guinea pig vas deferens were contradictory to this view.

It has been widely realized that autonomic nerve endings in the smooth muscle contain both agranular and granular vesicles. It will be of great value if one can clearly discriminate the adrenergic nerve fibers from the cholinergic ones according to the constituent synaptic vesicles in the axon.

Grillo and Palay\textsuperscript{11} have identified three types of granular vesicles in the rat: Type 1, a large structure about 840Å in diameter with a central droplet measuring about 470Å; Type 2, about 470Å in diameter with small central core consisting of a doublet of hollow cylinders measuring 130×340Å; Type 3, a vesicle about the same size as Type 2 in which a very dense central granule (90–280Å) occurs. But they did not classify these granulated vesicles from a standpoint of the cholinergic and adrenergic innervation.

Richardson\textsuperscript{2} found in the nerve endings of the rat vas deferens many granular vesicles of Grillo and Palay's Types 2 and 3. Also Merrillees et al.'s\textsuperscript{3} found the nerve endings of the guinea pig vas deferens to contain Type 2 granular vesicles and sparsely distributed Type 1 granular vesicles.

More recently, in the study of the innervation of the rabbit iris smooth muscle, Richardson\textsuperscript{12} concluded that granulated vesicles of Types 2 and 3 are the characteristic features of the adrenergic nerve endings and the uniformly populated agranular vesicles are of the cholinergic nerve endings.

There have been a considerable number of evidences that the densely stained substance in the synaptic vesicle is noradrenalin.\textsuperscript{13–16} A series of observations of Falck,\textsuperscript{17} Hamberger and Norberg,\textsuperscript{18} and Norberg\textsuperscript{19} demonstrated the adrenergic innervation of the several smooth muscle tissues by fluorescence microscopy. According to them, fluorescent adrenergic nerve fibers were found in the smooth muscle layers of the guinea pig vas deferens\textsuperscript{17} and the trigone area of the urinary bladder of the cat,\textsuperscript{18} but the fluorescent fibers were very sparse in the intestinal smooth muscles of the rat and the cat.\textsuperscript{19} Their results seem to be in good agreement with the present electron microscopic observations.

One of the interesting problems in the present observation is the nature of the large granular vesicles in the rabbit colon. Their large size (800–1,400Å) has not been observed in other smooth muscles, except those reported in the rat colon by Thaemert\textsuperscript{8} and in the sphincter pupillae of rabbit by Richardson.\textsuperscript{12} They might be an inhibitory adrenergic nerve fibers in the colon, but it is quite possible that they might be the excitatory cholinergic nerve fibers in this tissue, because this type of axons are exclusively present in the rabbit colon.

More detailed electron microscopic observations combined with histochemical
staining techniques and specific depletor drugs are required to clarify the nature of the granules in the terminal axons and further to resolve the complexity of the innervation of the smooth muscle tissue.

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References

1) Burnstock, G. & Holman, M.E. The transmission of excitation from autonomic nerve to smooth muscle. J. Physiol. (Lond.), 1961, 155, 115–133.

**Legends**

Fig. 1. A large bundle of unmyelinated nerve fibers with a Schwann cell nucleus (Sw) and cytoplasm in the rabbit colon smooth muscle layer. Three smooth muscle cells (M) are located on the right side of the figure. The axons contain a large number of mitochondria and neurofilaments but synaptic vesicles are sparse. Rabbit colon, OsO₄-KMnO₄ fixation. ×6,900.

Fig. 2. Many unmyelinated nerve fibers (Ax) are surrounded by Schwann cell cytoplasm and by collagen fibrils (C). Smooth muscle cells (M) with elongated nucleus (N) are separated from axons by a rather wide extracellular space where numerous collagen fibrils are present. Rat vas deferens, OsO₄ fixation. ×7,500.

Fig. 3. The nerve cells in Auerbach's plexus of rat ileum. A nerve cell is surrounded by many vesicle containing axons. At right, smooth muscle cells (M) are present being separated from the nerve cell by a wide extracellular space where fibroblasts (Fib) and collagen fibrils are present. The nucleus of a nerve cell (Nn) and the nucleus of a Schwann cell (Sw) are present. Rat ileum, KMnO₄ fixation. ×6,250.

Fig. 4. Schwann cell (Sw) enclosing some 15 axons (ZAx) is accompanied by a blood capillary (Cap) which contains erythrocytes in its lumen. Another blood capillary is seen at the upper left. Nerve bundles of this order are frequently accompanied with blood capillaries. Rabbit colon, OsO₄-KMnO₄ fixation. ×5,750.

Fig. 5. At the center of the figure is a single axon (Axs) attached closely to a smooth muscle cell. The synaptic vesicles in the axons are not clearly discriminated in this magnification. The nucleus (N) of a smooth muscle cell is present. Guinea pig urinary bladder, OsO₄-KMnO₄ fixation. ×6,250.

Fig. 6. An aggregate of axons (Ax) encircled by Schwann cell cytoplasm (Sw). Note the fine pinocytotic vesicles (P) beneath the smooth muscle membrane where the axons free from Schwann cell coat is opposing. The nucleus (N) of a smooth muscle cell is present at the upper left. Rabbit colon, OsO₄-KMnO₄ fixation. ×5,500.

Fig. 7. An aggregate of myelinated nerve fibers in the center of the unmyelinated nerve fiber bundle. The significance of this myelinated nerve fibers is unknown. Guinea pig taenia coli, OsO₄ fixation. ×5,000.

Fig. 8. Four axons with a Schwann cell nucleus (Sw) and cytoplasm seen in a relatively large extracellular space. Densely stained granular vesicles are seen in the lower axon. Guinea pig taenia coli, KMnO₄ fixation. ×17,500.

Fig. 9. Many terminal axons present in a narrow intercellular space between two smooth muscle cells (M). The axons are free from Schwann cell cytoplasm. Guinea pig taenia coli, KMnO₄ fixation. ×7,000.

Fig. 10. Another illustration the innervation of the taenia coli of the guinea pig. The axons contain mainly agranular vesicles. Note that the axons are not closely attached to a smooth muscle cell (M). The spacing is measured to be about 1,300 Å. Guinea pig taenia coli, KMnO₄ fixation. ×15,000.

Fig. 11. Five axons in close contact with a single smooth muscle cell (M). The contact area of these axons are free from Schwann cell cytoplasm but the external lamina still exists in this space. Rat ileum, KMnO₄ fixation. ×12,500.
Fig. 12. A cross section of a bundle of unmyelinated nerve fibers in the smooth muscle layer of rabbit colon. The uppermost axon contains characteristically large granular vesicles (V). Rabbit colon, OsO₄-KMnO₄ fixation. ×12,500.

Fig. 13. Most of the axons in this nerve bundle contain large granular vesicles. Smooth muscle cell (M) and collagen fibrils (C) are present. Rabbit colon, OsO₄-KMnO₄ fixation. ×15,000.

Fig. 14. An enlargement of a part of the Fig. 7, showing the abundant pinocytotic vesicles (P) beneath the muscle cell membrane opposing the naked axon. Note that the axons contain large granular vesicles (V). Rabbit colon, OsO₄-KMnO₄ fixation. ×6,750.

Fig. 15. Longitudinally sectioned axons are enclosed in Schwann cell cytoplasm (Sw). Note the presence of rich pinocytotic vesicles beneath the muscle cell membrane opposing the axon and Schwann cell cytoplasm. Rabbit colon, OsO₄-KMnO₄ fixation. ×8,500.

Fig. 16. Two axons in the figure contain large granular vesicles. Schwann cell cytoplasm (Sw) encloses four axons in the figure. Pinocytotic vesicles (P) are present on the muscle cell membrane. Rabbit colon, OsO₄-KMnO₄ fixation. ×15,000.

Fig. 17. Note the abundant presence of the large granular vesicles in the obliquely sectioned axon. A multi-vesiculated synaptic vesicle (V) is present at the lower right. Rabbit colon, OsO₄-KMnO₄ fixation. ×15,000.

Fig. 18. Myelinated nerve fibers (ML) are simultaneously present with unmyelinated nerve fibers in the serosal coat of the vas deferens. Schwann cell cytoplasm (Sw) encloses unmyelinated nerve fibers. Abundant collagen fibrils (C) are present around the axon. Rat vas deferens, OsO₄ fixation. ×12,500.

Fig. 19. Three axons (Ax) in a relatively large extracellular space of guinea pig vas deferens longitudinal muscle layer. The greater part of the synaptic vesicles in these axons are granular vesicles of average diameter 300–520 Å. Guinea pig vas deferens, KMnO₄ fixation. ×27,000.

Fig. 20. Single axon with two mitochondria and abundant granular vesicles found in a narrow space between the smooth muscle cells (M) of the guinea pig vas deferens. KMnO₄ fixation. ×30,000.

Fig. 21. Single axon in close contact with a muscle cell (M). This axon contains two mitochondria and synaptic vesicles of granular type. A small quantity of Schwann cell cytoplasm (Sw) is attached to the axon. Guinea pig vas deferens, KMnO₄ fixation. ×30,000.

Fig. 22. Single axon buried deep in a groove of a smooth muscle cell (M). Pinocytotic vesicles (P) occur on the muscle cell membrane at the bottom of the groove. Large synaptic vesicles (V) are present in the axon. Guinea pig vas deferens, KMnO₄ fixation. ×36,000.

Fig. 23. Single axon buried deep in a groove of a smooth muscle cell (M). In this case, the vesicles (P) occur on the adjoining smooth muscle cell membrane not directly related to the axon. Guinea pig vas deferens, KMnO₄ fixation. ×36,000.

Fig. 24. Two terminal axons (Ax) in close contact with smooth muscle cell (M) of seminal vesicle. An unidentified lamellar structure (In) is present between the axons. At lower left is the nucleus (N) of a smooth muscle cell. Guinea pig seminal vesicle, OsO₄-KMnO₄ fixation. ×12,500.

Fig. 25. High power magnification of the synaptic vesicles in an axon of the seminal vesicle preparation. In this axon small agranular vesicles are predominantly found than granular vesicles. Rich pinocytotic vesicles (P) are present in the process of a smooth muscle cell. Guinea pig seminal vesicle, OsO₄-KMnO₄ fixation. ×30,000.

Fig. 26. Single axon (Ax) in the intercellular space of three smooth muscle cells (M) of the urinary bladder. In this axon, mitochondria are abundant but synaptic vesicles
are sparse. Guinea pig urinary bladder, OsO₄ fixation. ×23,000.

Fig. 27. Single axon (Ax) buried in a groove of a smooth muscle cell of urinary bladder. This axon mainly contains granular vesicles. Smooth surfaced endoplasmic reticulum (ER) are seen in the muscle cell at the left. Guinea pig urinary bladder, KMnO₄ fixation. ×34,500.

Fig. 28. Single axon (Ax) in the complicated processes of muscle cell. Pinocytotic vesicles and endoplasmic reticulum (ER) are seen beneath the muscle cell membrane opposing the axon. Guinea pig urinary bladder, OsO₄ fixation. ×25,000.

Fig. 29. A longitudinally sectioned terminal axon (Ax) forming a synaptic knob present at the center of the figure. Muscle cell membrane opposing the axon contains rich pinocytotic vesicles, endoplasmic reticulum (ER) and mitochondria. Rat ureter, KMnO₄ fixation. ×8,500.