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A STUDY OF EXCITATORY NEUROMUSCULAR TRANSMISSION IN THE BOVINE TRACHEA

BY A. R. CAMERON* AND C. T. KIRKPATRICK†
For the Physiology Department, The Queen's University of Belfast, Northern Ireland

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SUMMARY

1. The excitatory innervation of bovine tracheal smooth muscle has been studied with the sucrose-gap apparatus.

2. Single 2 ms electrical stimuli applied to the whole tissue excited intrinsic nerves, and produced a small transient depolarization of the smooth muscle, the excitatory junction potential (e.j.p.). The e.j.p. caused a twitch-type contraction; twitches and e.j.p.s summated during repetitive stimulation but facilitation was not observed, and action potentials were never elicited.

3. The effects of electrical stimulation could be abolished by atropine (5×10^{-7} mol/l) and augmented by neostigmine (4×10^{-6} mol/l), and were mimicked by exogenous acetylcholine (1·0 μg/ml).

4. With the electron microscope, the density of innervation was found to be low (one axon per ninety smooth muscle cells). Axons were found in small groups in the clefts between bundles of cells, but no axons penetrated within the muscle bundles. Naked axon varicosities containing agranular vesicles were seen, but no axon approached within 200 nm of a smooth muscle cell.

5. It is difficult to reconcile the sparsity of innervation with the dependence of the tissue on nerve excitation to initiate activity.

INTRODUCTION

The respiratory airways receive an excitatory bronchomotor innervation from the parasympathetic system via the vagus nerve, which might have an important role in the mediation of bronchospasm. There may also be an inhibitory innervation from the sympathetic system. Although the effects of the neuro-transmitters on the state of contraction or relaxation

* M.R.C. Postgraduate Student.
† Present address: Physiological Department, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL.

of the smooth muscle are well known (McDougal & West, 1953; Widdicombe, 1963), it is not yet certain how these effects are produced at the cellular level. This paper describes some of the changes in the membrane potential of tracheal smooth muscle cells during the excitatory action of the autonomic nerves, and during the action of the putative neurotransmitter. It describes also some features of the anatomical relationship between the smooth muscle cells and the intrinsic autonomic nerves; an attempt is made to correlate the structure with the electrophysiological behaviour of the tissue. Some of these results have been communicated briefly at meetings of the Physiological Society (Cameron & Kirkpatrick, 1976).

METHODS

Segments of thoracic trachea were obtained from cattle at the abattoir, and strips of the smooth muscle layer were isolated as described by Kirkpatrick (1975). The strips were either processed histologically for examination with the electron microscope, or were mounted in the sucrose-gap apparatus.

Sucrose-gap technique. The tracheal muscle strips were mounted in a single-sucrose-gap apparatus (Bülbring & Burnstock, 1960) consisting of a series of tubes which divided the tissue into three segments: a central segment perfused with isotonic deionized sucrose (98 g/l), an 'active' region perfused with Krebs solution or a test solution at 38 °C, and (at the opposite end) a depolarized region perfused with isotonic K_2SO_4 (18 g/l). The purpose of the sucrose was to increase the extracellular resistance, so that potential differences could be measured between the ends of the tissue; contact with the 'active' and depolarized region was made with agar-KCl (2 M) salt bridges and Ag/AgCl electrodes, and the potential difference was measured with a high-impedance amplifier.

The segment of tissue in the 'active' region was passed through a pair of platinum ring electrodes, so that electrical stimuli could be applied. By using short pulses (duration < 2 ms), the intrinsic nerves could be stimulated without causing direct activation of the smooth muscle fibres (which require a stimulus duration of > 20 ms). The 'active' end of the muscle strip was attached to an isometric force transducer for recording of the contractile activity of the muscle.

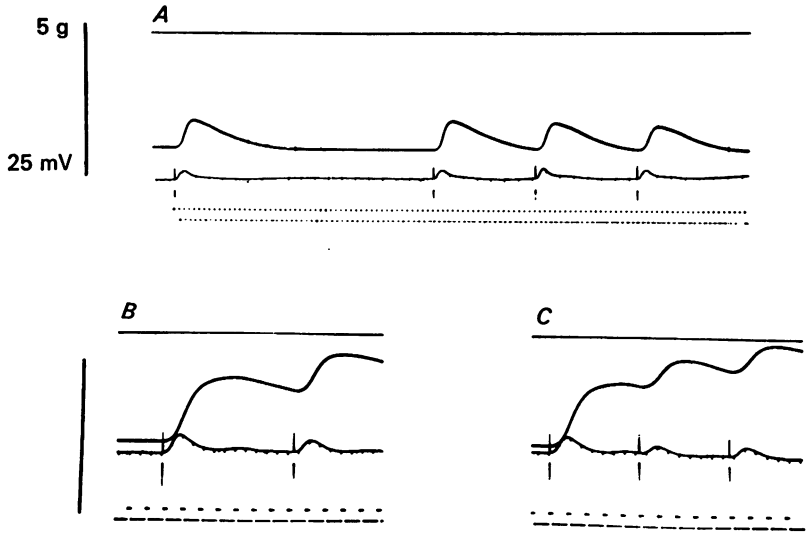
Electron microscopy. Strips of tracheal muscle were suspended either from stainless-steel pins, with 250 mg weights attached to the lower end to form an isotonic load, or were mounted on a stainless-steel wire frame under isometric conditions. They were immersed in vials containing oxygenated Krebs solution at 38 °C for at least 1 h, and were then fixed with 5% glutaraldehyde in cacodylate (0·125 mol/l) buffer at room temperature for 2½ h, and post-fixed in osmium tetroxide. They were block-stained in aqueous uranyl acetate, dehydrated in graded alcohols and propylene oxide, and embedded in epoxy resin (Durcupan, Fluka) for sectioning with a glass knife on an ultramicrotome (L.K.B.). Transverse and longitudinal sections were cut at 60 nm, mounted on copper grids, stained with lead citrate and examined with an EM6B electron microscope (A.E.I.).

Solutions and drugs. The normal Krebs solution had the following composition (mmol/l): NaCl, 120; KCl, 5·9; $NaHCO_3$, 15·5; NaH_2PO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 2·5; glucose, 11·5. The solution was gassed with 5% CO_2 in O_2 and had a pH of 7·4 at 38 °C.

The following drugs were used: acetylcholine hydrochloride (Lamatte et Boinot, Paris, or Sigma); atropine sulphate (Evans Medical); neostigmine hydrobromide (Roche).

RESULTS

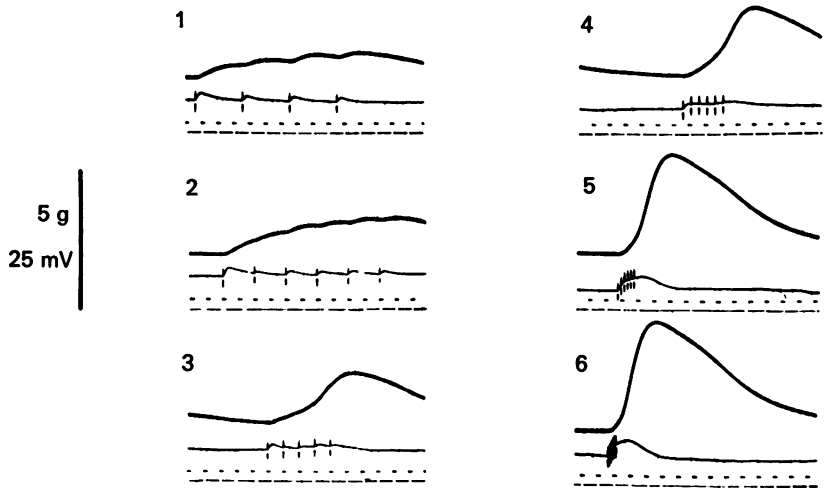
In the absence of stimulation, the potential record and tension of tracheal smooth muscle were constant; there was no spontaneous activity. A single 2 ms pulse applied through the platinum ring electrodes in the 'active' region of the sucrose-gap apparatus produced a wave of depolari-



Text-fig. 1. *A*, effect of single pulses (2·0 ms duration) on mechanical (upper trace) and electrical (lower trace) activity of tracheal smooth muscle. Top line is a reference line; lowest trace is time marker (1 s). *B*, *C*, summation of contraction (upper trace) while e.j.p.s on electrical record (lower trace) remain discrete. Vertical lines on potential record which precede e.j.p.s (excitatory junction potential) are stimulus artifacts.

zation (the excitatory junction potential, e.j.p.) which was followed by a brief twitch-type contraction of the muscle strip (Text-fig. 1*a*). The amplitude of the e.j.p. recorded by the sucrose-gap technique was in the range 0·1–4 mV, the actual size depending upon the recording conditions of the particular experiment. The latency between the stimulus artifact and the onset of the e.j.p. was in the range 100–250 ms; this latency is due, in part, to the conduction time down the fine nerve terminals from the stimulus site to the interface between Krebs solution and sucrose, a distance of 3 mm. The time to peak of the e.j.p. was about 800 ms and the time constant of decay was about 1500 ms.

The amplitude of the e.j.p. and contraction were graded according to the strength of the stimulating pulse; in most cases the maximum output available from the stimulator (isolated constant current unit powered by 45 V battery) was used, but detectable responses could be obtained using pulses 0·25 × max.



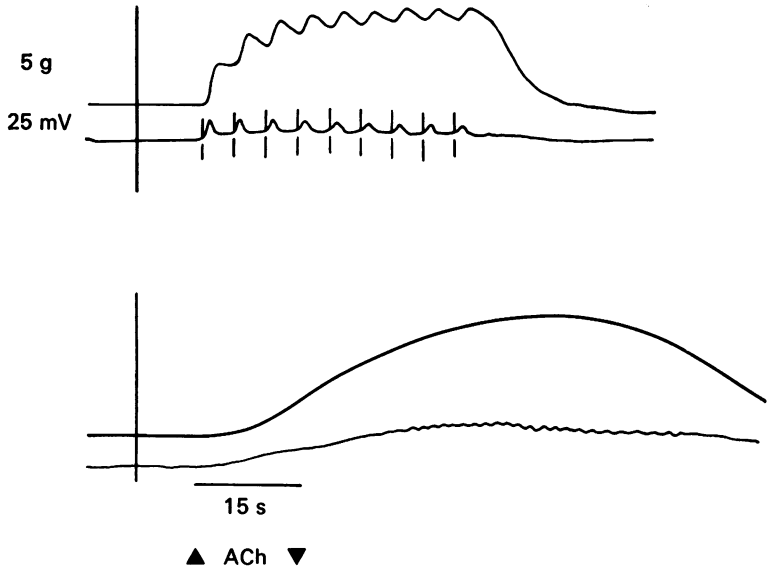
Text-fig. 2. Effects of repetitive electrical stimulation (2·0 ms pulses) on mechanical (upper traces) and electrical (lower traces) activity of tracheal muscle. Lowest trace in each record is time marker (1 s). Frequencies of stimulation: 1, 0·2 Hz; 2, 0·5 Hz; 3, 1·0 Hz; 4, 2·0 Hz; 5, 5·0 Hz; 6, 10 Hz. Tension and voltage calibration apply to all records.

The duration of the twitch was much longer than the duration of the e.j.p. and it was possible, by applying stimuli even at fairly wide intervals, to produce summation of the contractions even though the e.j.p.s remained separate (Text-fig. 1*b*, *c*). As the frequency of stimulation was increased, fusion of the contractions occurred to produce a tetanus, and at frequencies above 1 Hz there was summation and finally fusion of the e.j.p.s as well, to produce a smooth depolarization (Text-fig. 2). With increasing frequency of stimulation but with a constant number of stimuli, the amplitudes of the depolarization and the tetanus increased, with a maximum around 50 Hz. At no frequency of stimulation was facilitation of the e.j.p.s observed; in fact, at all frequencies higher than 0·5 Hz there was depression of the e.j.p.s following the first in a train.

It was assumed that the e.j.p. and contraction were caused by the release of acetylcholine from the intrinsic parasympathetic nerves. In order to test this assumption, standard pharmacological tests were applied. Thus

atropine (5×10^{-7} mol/l) reduced or abolished both the e.j.p. and the contraction and neostigmine (4×10^{-6} mol/l) augmented the amplitude of both the e.j.p. and the contraction.

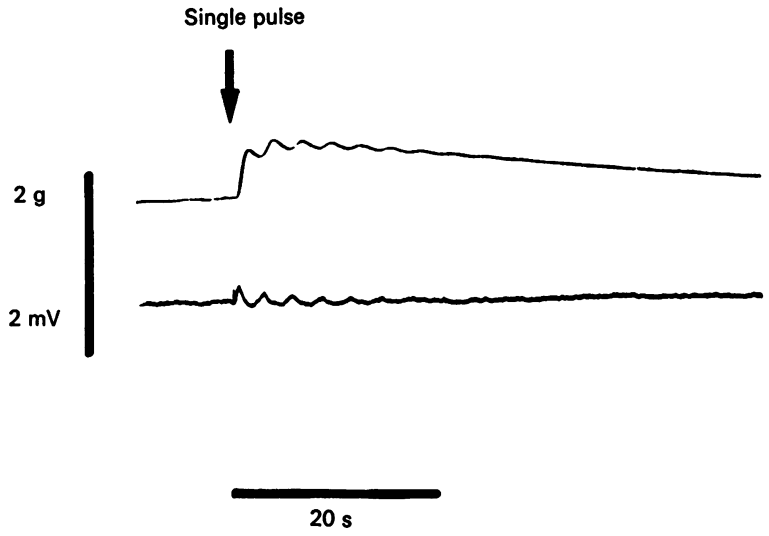
When exogenous acetylcholine was added to the Krebs solution perfusing the tissue, it produced a depolarization and contraction of the same order of magnitude as those produced by repetitive electrical stimulation of the intrinsic nerves (Text-fig. 3). The onset of depolarization and contraction were, of course, much slower for exogenous acetylcholine than for nerve



Text-fig. 3. Upper record: effect of electrical stimulation (2 ms pulses at 0·2 Hz) on mechanical (upper trace) and electrical (lower trace) response of tracheal muscle. Lower record: effect of acetylcholine (ACh; 1 μg/ml) applied during period indicated by filled triangles. Tension, potential and time traces apply to both records, which were taken from the same specimen.

stimulation, as the drug would have to diffuse into and through the tissue, whereas the neuro-transmitter is released close to the muscle. When the depolarization due to acetylcholine had reached a level of 12 mV, a series of slow, small-amplitude oscillations in the potential trace were recorded; there were no corresponding oscillations in the tension trace. This ability of acetylcholine to induce oscillatory behaviour in the membrane potential, if it acts for long enough, was further illustrated by the occasional finding of slow oscillations, following the e.j.p., when neostigmine was present to prevent the breakdown of acetylcholine by cholinesterase (Text-fig. 4). In such cases the potential oscillations were often accompanied by fluctuations in tension.

In no case was it possible, either by electrical stimulation or by the application of acetylcholine or neostigmine, to produce an action potential in tracheal smooth muscle. The contractions were not dependent upon action potentials, but occurred when the membrane was depolarized. The dependence of the amplitudes of contraction and depolarization upon stimulus frequency are shown in Text-fig. 5*a*, and the relationship between contraction and depolarization are shown in Text-fig. 5*b*.

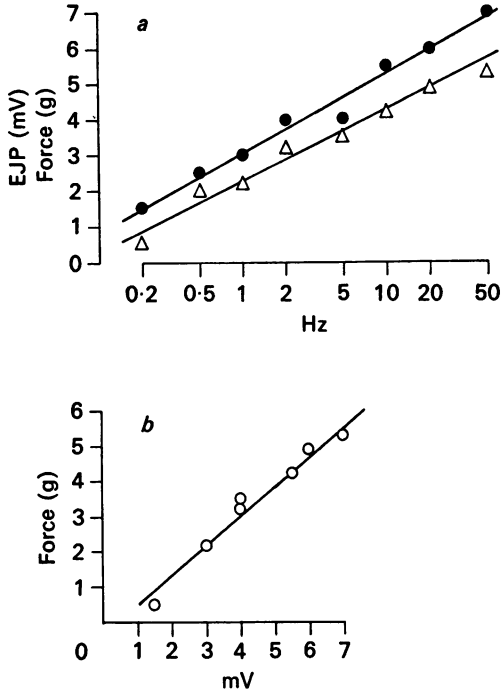


Text-fig. 4. Response of tracheal muscle to single 2 ms pulse, in presence of neostigmine (10^{-5} mol/l). Upper trace: mechanical record. Lower trace: electrical record. Note oscillatory electrical activity following the EJP: this is associated with prolonged, oscillating concentration.

In a smooth muscle which has no spontaneous activity, and which is dependent on its nerves to initiate electrical and mechanical activity, one would expect that the nerve supply should be very dense, that the nerve terminals should be very close to the smooth muscle cells, and that each smooth muscle cell should be in close relation to one or more nerve terminals. The bovine trachea was examined with the electron microscope to see if this were the case.

The smooth muscle was arranged in fairly large bundles of cells, and the autonomic nerves were found in the clefts between bundles (Pls. 1 and 2). No autonomic nerves were discovered within the cell bundles, and the nerve terminals did not approach the smooth muscle cells very closely. The autonomic nerves were arranged as small bundles of axons with varicosities along their length; from time to time a varicosity was found which was denuded of its Schwann cell covering, and this region, which

was often packed with agranular vesicles, presumably containing acetylcholine, came fairly close to a group of smooth muscle cells (Pl. 3). At no time did any varicosity approach closer to a smooth muscle cell than 200 nm, and the majority of axon varicosities were at least 650 nm away.



Text-fig. 5 (*a*) relationship between amplitude of depolarization or summed e.j.p.s (○) or isometric twitch (△) and frequency of stimulation (log scale). (*b*) relationship between force of contraction of tracheal muscle strip and amplitude of depolarization recorded in sucrose-gap apparatus (re-plotted data of (*a*)). Points represent data from a representative expt.; similar results were obtained in five expts.

The size and consistency of the vesicles in axon varicosities are often assumed to indicate the nature of the transmitter in the nerves; agranular vesicles are taken to be cholinergic, and small granular vesicles are taken to be adrenergic (Burnstock, 1970). All of the axon varicosities examined in bovine tracheal muscle were of the cholinergic type, with agranular vesicles; no dense-cored adrenergic vesicles were discovered in association with the smooth muscle.

For a quantitative estimate of the density of innervation, pieces of muscle from six tracheae were fixed; from each block, five sections were cut from widely separated sites, and one entire grid square (mean area

4100 μm^2) from each section was examined. Every muscle cell profile and each axon was counted in thirty such grid squares, and the packing density of smooth muscle cells and nerves was calculated.

A total of 9755 smooth muscle cells were counted; these had a packing density of 78,000 cell/mm^2 (S.D. of observations = 30,000). A total of 353 axons were seen; these had a packing density of 2800 axons/mm^2 (S.D. of observations = 2500). The crude ratio of cells to axons was thus 28:1; however, many of the axons were cut in their pre-terminal region, i.e. they were found in large or small bundles or trunks which were totally or partially surrounded by perineurium or interstitial cells. Only 100 of the 353 axons were in the final or terminating part of their course, where the varicosities become partially denuded of Schwann cell covering, and where other extrinsic coverings are absent. Thus the ratio of smooth muscle cells to axons which are effectively innervating the muscle is 97:1 instead of 28:1.

The axons were usually arranged in small groups, although many appeared singly. The size of nerve groups ranged from one to twenty-one axons, with a mean of 3·5 (S.D. of observations = 2·9) axons per bundle. A total of 100 bundles of isolated axons were found, of which forty-two were in the terminating part of their course.

Only twenty-nine of the 353 axons approached within 1000 nm of a smooth muscle cell, and of these only four approached within 250 nm. Thus, the effective innervation of tracheal smooth muscle is very sparse indeed.

In order to study cell-to-cell connexions in the smooth muscle, sections from the same six blocks of tissue were photographed at higher magnification. A total of 502 cell profiles were examined, and 236 cell-to-cell contacts were found. Of these, forty were true nexuses (Henderson, 1975), 143 were point contacts and fifty-three were close approaches between the membranes of adjacent cells; at these close approaches, although the membranes showed no specialization, the basement membrane was absent between the cells.

DISCUSSION

Changes in the membrane potential of tracheal smooth muscle in response to nerve stimulation have been demonstrated, using the sucrose-gap technique. The potential change, which is called the excitatory junction potential (Burnstock & Holman, 1960), precedes the onset of contraction and is therefore not an artifact produced by movement of the muscle; it is not an electrical artifact, as the stimulus artifact seen in the records is very short, and the e.j.p. persists without change when the stimulus polarity is reversed. We believe it is a genuine synaptic potential produced by neuro-transmitter liberated from the intrinsic nerves.

The potential changes may not represent the absolute changes which would be recorded intracellularly using micro-electrodes, since the sucrose-gap technique records the summed activity of a large number of interconnected smooth muscle cells near the Krebs solution/sucrose interface, rather than the activity of a single cell. The technique indicates only a proportion of the true membrane potential, due to the variable amount of short-circuiting through the sucrose stream (see Coburn, Ohba & Tomita, 1975, for a critique of the sucrose-gap technique), and the summing process may explain the long latency and prolonged duration of the e.j.p., although long latencies are a feature of muscarinic transmission at a number of sites (Purves, 1976; Gillespie, 1962; Holman, 1970; Ohashi & Ohga, 1967).

The transmitter involved in producing the e.j.p. appears to be acetylcholine, as the e.j.p. is abolished by atropine and augmented by neostigmine. The depolarization and contraction during nerve stimulation can be mimicked by exogenous acetylcholine, although the identity of the actions has not been established.

In many smooth muscles, the e.j.p. is not sufficient by itself to initiate contraction. Thus, in the guinea-pig vas deferens (with a sympathetic innervation; Burnstock & Holman, 1960, 1961) and the chick oesophagus (with a muscarinic parasympathetic innervation; Ohashi & Ohga, 1967) the e.j.p. is a subthreshold phenomenon, and it is necessary for several e.j.p.s to summate temporally or spatially to produce action potentials which trigger contractions. However, in tracheal muscle each e.j.p. initiated a contraction, which was related to the amplitude of depolarization; it was not possible to produce an action potential by nerve stimulation, and action potentials were not necessary for contraction. The production of graded contractions in response to graded depolarizations, without the need for spikes, is seen in the smooth muscle of the anococcygeus (Creed, Gillespie & Muir, 1975) and the stomach (Vladimirova, 1976) and is also characteristic of neuromuscular transmission in many invertebrates (Fatt & Katz, 1953) and in amphibian slow muscle (Kuffler & Vaughan Williams, 1953).

The production of oscillations in the membrane potential by application of acetylcholine is a new finding, though depolarization by this agent has been previously described for tracheal muscle (Stephens & Kroeger, 1970; Kirkpatrick, 1975). Oscillations have been described during the response to histamine (Kirkpatrick, 1975) and serotonin (Coburn & Yamaguchi, 1975); it is likely that any agent which depolarizes the membrane into a particular range of potentials could initiate such oscillations, and potential-dependent slow waves have been recorded in ileal muscle (Bolton, 1971) and in stomach muscle (Ohba, Sakamoto & Tomita, 1975).

Most reports of neuro-muscular transmission in smooth muscle have

emphasized the facilitation of e.j.p.s during a train of stimuli (e.g. vas deferens: Burnstock, Holman & Kuriyama, 1964; Chicken oesophagus: Ohashi & Ohga, 1967; anococcygeus; Creed *et al.* 1975). We have not seen facilitation in tracheal muscle; in fact, we have observed depression regularly in the second and subsequent e.j.p.s in a train. This failure to observe facilitation is not an artifact of the sucrose-gap technique, as Ohashi & Ohga (1967) were able with this method to demonstrate facilitation quite clearly in the chick oesophagus, which also has a cholinergic innervation. We do not know why facilitation fails to occur in the trachea, nor do we know why depression of e.j.p.s is observed.

Several previous ultrastructural and histochemical studies have been made of mammalian tracheobronchial smooth muscle (e.g. Altenahr, 1965; Blumke, 1968; Mann, 1971; Nakamori, 1971; Rikimaru & Sudoh, 1971; Silva & Ross, 1974; Suzuki, Morita & Kuriyama, 1976) and there has been considerable variation in the description of the pattern of innervation. There are species differences (Mann, 1971), and even a variation in density of adrenergic innervation between different parts of the trachea of the same animal (Coburn & Tomita, 1973). The previous reports have often included strong statements on the density or otherwise of the innervation (e.g. Silva & Ross, 1974) but none have contained quantitative estimates of the density. The previously published micrographs have concentrated on the nerves, and few have included large regions of muscle in order to place the nerves in perspective.

We have photographed a large number of representative areas of tracheal smooth muscle, in order to arrive at a quantitative ratio between the number of muscle cells and the number of axons, and have demonstrated that the innervation of the muscle, at least in cattle, is sparse. No nerves lie within the bundles of muscle cells, and no axon varicosities approach the muscle cells really closely. This is surprising; most smooth muscles which depend upon nerves to initiate excitation have a very dense innervation, with many close neuro-muscular approaches (e.g. vas deferens: Bennett & Merrillees, 1966; intrinsic eye muscles: Gabella, 1976). The type of innervation, present in tracheal muscle, is found more often in muscles which exhibit spontaneous activity (e.g. intestinal muscle: Bennett & Rogers, 1967), and whose nerves serve to *modulate* the activity rather than to *initiate* it.

It is perhaps surprising that e.j.p.s can be recorded at all in tracheal muscle, in view of its sparse innervation and therefore diffuse liberation of transmitter. The portal vein has a similar diffuse pattern of innervation, and in this preparation nerve stimulation in a sucrose-gap chamber produces depolarization and an acceleration of spike discharge, but no individual e.j.p.s can be distinguished (Holman, Kasby, Suthers & Wilson, 1968).

The probable sequence of events during neuro-muscular transmission in tracheal smooth muscle is as follows: action potentials in the post-ganglionic parasympathetic nerve fibres cause release of acetylcholine from the axon varicosities into the clefts, and produce depolarization of the more peripheral cells. The cells in smooth muscle are interconnected by low-resistance contacts, and the space constant of tracheal muscle is fairly long, 1·5–3·5 mm (Kroeger & Stephens, 1975; Suzuki *et al.* 1976), so that even fairly small potential changes in these outer cells could probably be conduced deep into the bundle without much decrement, causing excitation and contraction of the whole bundle.

We have produced electrophysiological evidence in support of a cholinergic excitatory innervation for tracheal smooth muscle; we have thus confirmed what has long been suspected from mechanical and pharmacological evidence (Widdicombe, 1963). We have made no mention, so far, of possible inhibitory transmission, as our evidence for this is still incomplete.

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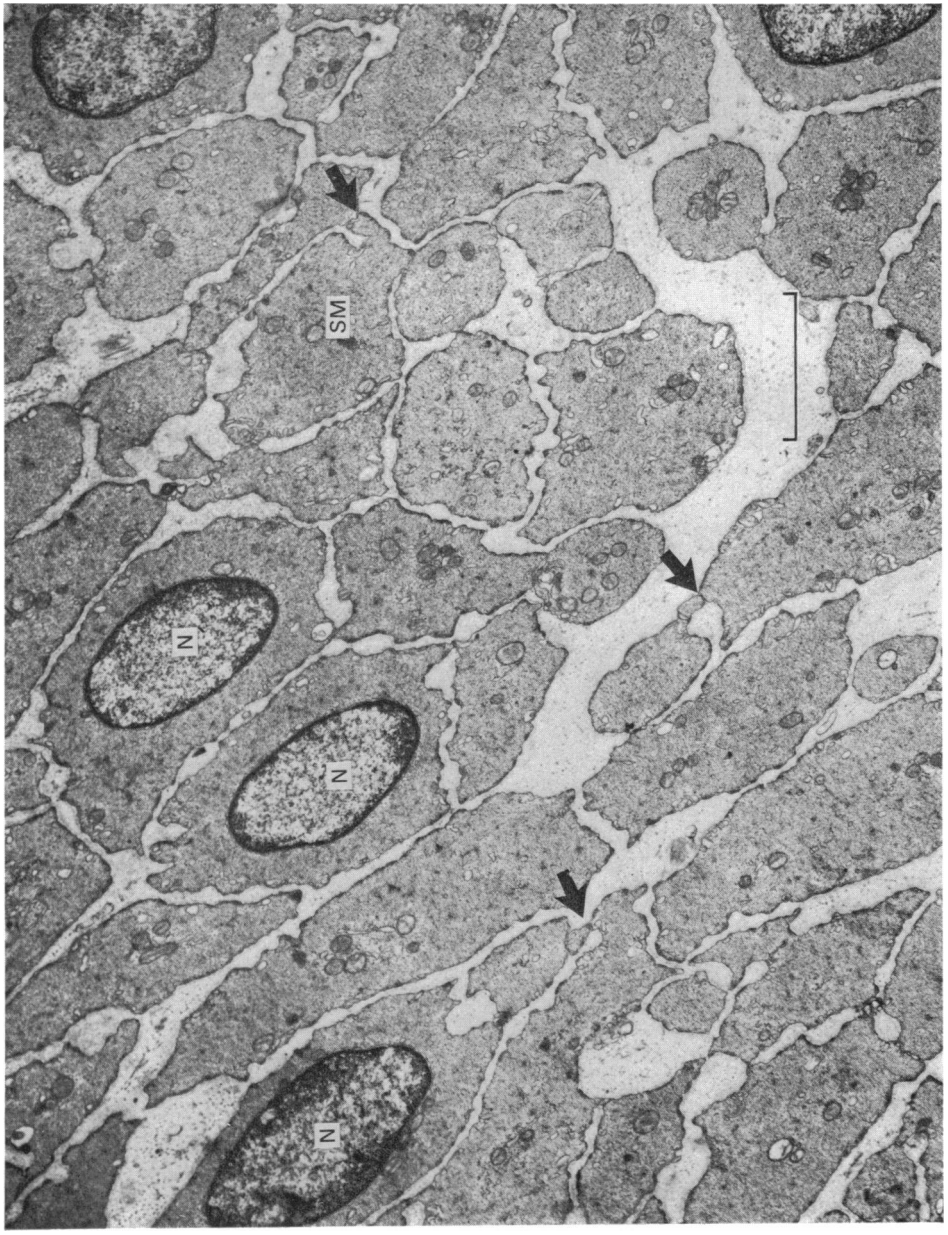
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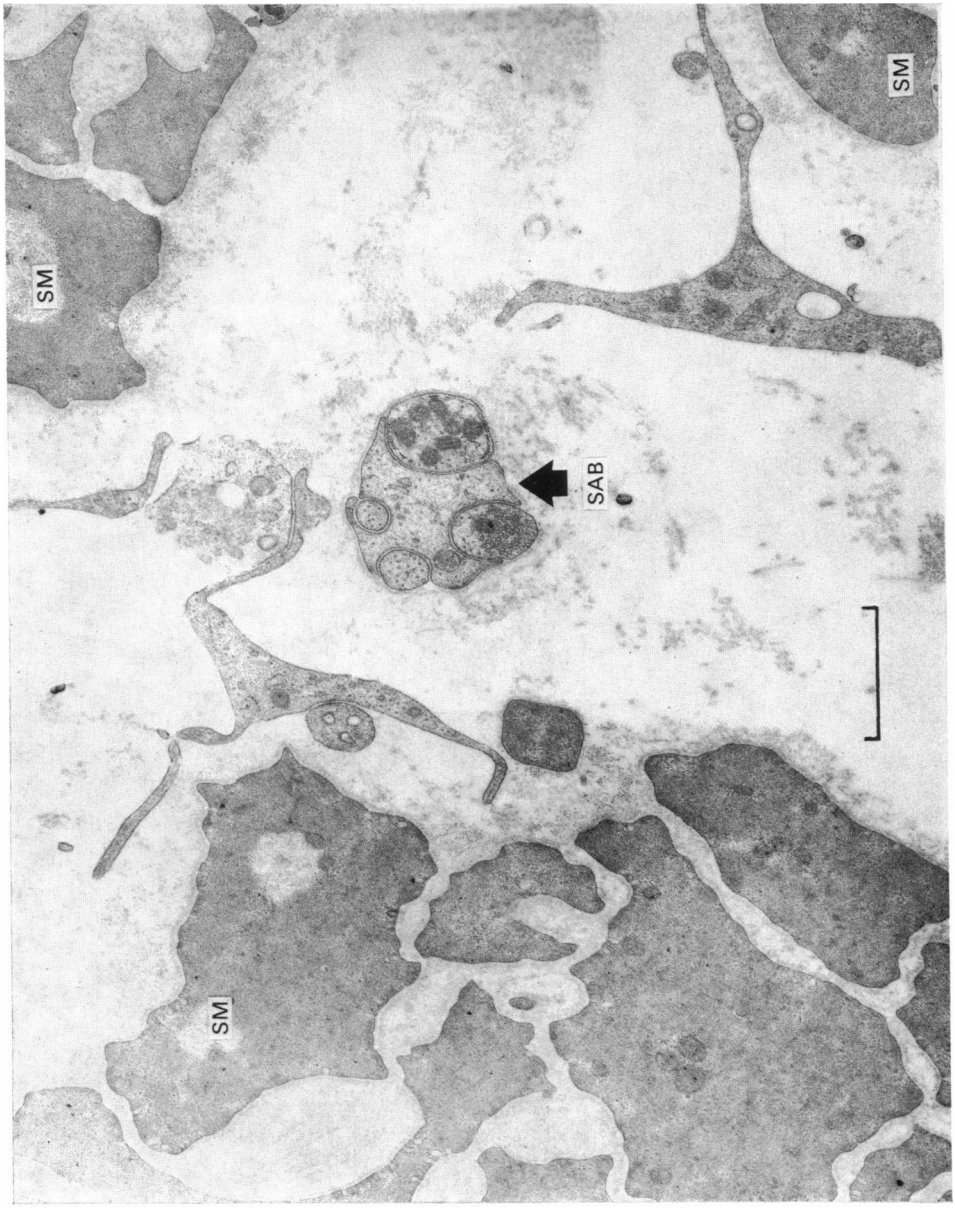

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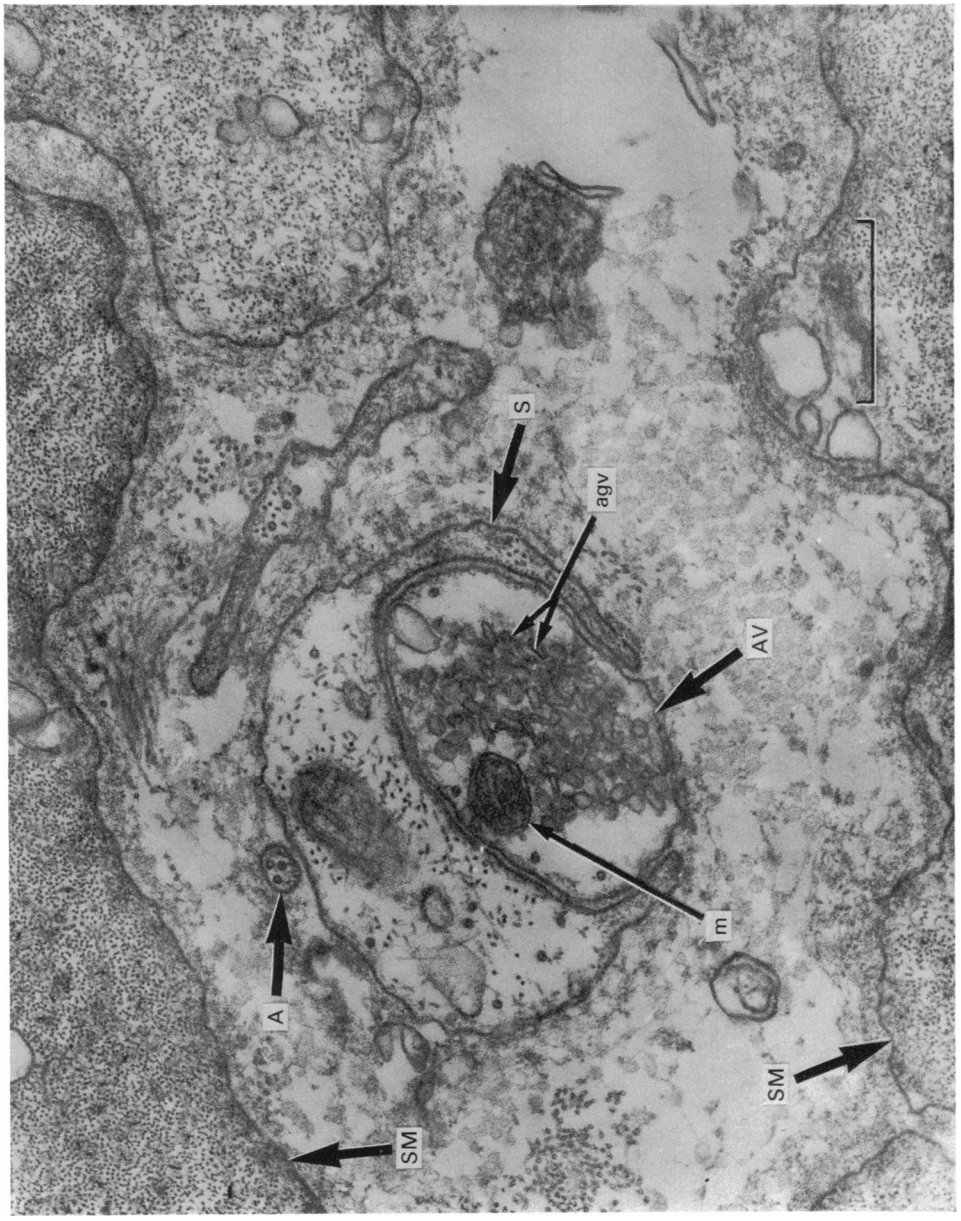
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EXPLANATION OF PLATES

PLATE 1

Low-power (× 12,000) electron micrograph of bovine tracheal muscle in transverse section. Note absence of nerves within muscle bundle. SM, smooth muscle cells. N, nucleus. Arrows: cell-to-cell contacts. Calibration bar 2 μm.

PLATE 2

Electron micrograph (× 22,500) showing small bundle in the cleft between smooth muscle bundles. SM, smooth muscle bundles. SAB, small axon bundle. Calibration bar 1 μm.

PLATE 3

High-power (× 60,000) micrograph showing a small nerve bundle near a group of smooth muscle cells. SM, smooth muscle cells. A, axon, in intervaricose region. AV, axon varicosity, containing agranular vesicles (agv). m. mitochondrion. S, Schwann cell. Note the region of the axon varicosity which is devoid of Schwann cell. Calibration bar 0·5 μm