Journal of Physiology (1992), **456**, *pp.* 557–574
With 12 figures
Printed in Great Britain

MULTIPLE MOTOR PATHWAYS TO SINGLE SMOOTH MUSCLE CELLS IN THE FERRET TRACHEA

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(*Received 29 August 1991*)

SUMMARY

1. We investigated the distribution and characteristics of motor pathways to individual smooth muscle cells activated by electrical stimulation of either, single nerves which enter the tracheal plexus (inlet nerves), or a longitudinal nerve trunk (LNT) located near the entrance of an inlet nerve into the plexus. Excitatory junction potentials (EJPs) were recorded using intracellular microelectrodes as an index of smooth muscle cell activation. In all experiments EJPs were completely blocked by tetrodotoxin and by atropine.

2. In smooth muscle fields located in the caudal direction from the point of inlet or LNT nerve stimulation, neural input decreased as a function of distance. There was evidence of a demarcated area innervated by neurons entering the plexus in one inlet nerve. In smooth muscle fields located in the rostral or transverse direction from the site of nerve stimulation, no such demarcated area could be identified.

3. Of the smooth muscle cells located within the innervated fields studied, 83–95% were activated following stimulation of a single inlet nerve or LNT. Evoked EJPs were similar in different innervated cells or units of electrically coupled cells located within the same 1 mm^2 'field'.

4. There was overlapping cholinergic motor input to single smooth muscle cells originating from neurons present in different inlet nerves or different neurons present in the same inlet nerve or region of the LNT. Multiple small step increases in the voltage used to stimulate a LNT resulted in three or four step increases in EJP amplitudes. This gives a minimal value for the number of motor pathways that can be activated by neurons in a region of LNT leading to a single smooth muscle cell.

5. Motor pathways to smooth muscle cells located in caudal and rostral fields ran initially in the LNT and exited in proximity to the smooth muscle cell studied.

6. Motor pathways used in transmitting signals to smooth muscle cells to different areas of trachealis muscle varied in their sensitivity to hexamethonium or curare. EJPs evoked in fields located in the caudal direction from the stimulating electrode were abolished by these drugs. Muscle cells located in different rostral fields showed EJPs that were either sensitive or resistant to these drugs.

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7. The rostral hexamethonium-resistant pathway ran initially in the LNT but it exited from the LNT several millimetres before reaching the level of the smooth muscle field innervated. This pathway followed stimulation frequencies up to 25 Hz. The final neuron in this pathway released acetylcholine and evoked EJPs were entirely inhibited by atropine. This pathway was not inhibited by capsaicin pretreatment.

8. After-hyperpolization neurons which run in the LNT may be involved in hexamethonium- or curare-sensitive motor pathways; these neurons are not components of the hexamethonium-resistant excitatory motor pathway to rostral smooth muscle cells.

9. Data showed a pattern of overlapping, convergent innervation to smooth muscle cells. Up to four motor pathways can be activated by recruiting different neurons in a single inlet nerve or region of the LNT. Motor pathways activated by stimulating different inlet nerves supply the same smooth muscle cell. The motor pathway supplying excitatory input to smooth muscle cells located in the caudal direction from the point of stimulation is a classical pathway in the sense that it traverses a nicotinic cholinergic synapse, and postganglionic neurons supply acetylcholine to smooth muscle cells. A motor pathway, running in the rostral direction from the point of nerve stimulation, which does not traverse nicotinic synapses but releases acetylcholine at the myoneural junction was characterized.

INTRODUCTION

Stimulation of the vagus nerve causes bronchoconstriction in all mammalian species (Olson, Colebatch, Mebel, Nadel & Staub, 1965; Woolcock, Macklem, Hogg, Wilson, Nadel, Frank & Brain, 1969; Cabezas, Graf & Nadel, 1971; Blackman & McCaig, 1983; Leff, Munoz, Tallet, David, Cavigelli & Garrity, 1985; McWilliam & Gray, 1990). The concept that preganglionic motoneurons synapse within peripheral airway parasympathetic ganglia, and that postganglionic cholinergic excitatory neurons innervate smooth muscle cells, is well established (reviewed by Coburn, 1989).

Vagal motor nerves enter a plexus present on the serosal surface of airway muscle (parasympathetic nerve and ganglion plexus). Some of the electrophysiological properties of airway ganglion cells have been described in the cat (Mitchell, Herbert, Baker & Basbaum, 1987), rat (Allen & Burnstock, 1990) and ferret (Baker, Basbaum, Herbert & Mitchell, 1983; Cameron & Coburn, 1984). To our knowledge the only detailed studies of the organization and structure of this plexus have been performed on the ferret trachea. In this species the paratracheal nerve–ganglion plexus contains one or two large nerve trunks that run longitudinally the entire length of the trachea (longitudinal nerve trunks, LNTs) and superficial and deep plexi that cover the posterior surface of the trachea (Cameron & Coburn, 1984; Baker, McDonald, Basbaum & Mitchell, 1986; Coburn & Kalia, 1986). Six to eight inlet nerves, originating in the vagus nerve, enter the plexus. Figure 1 illustrates the anatomy of the ferret paratracheal nerve–ganglion plexus.

There are multiple ganglia associated with the LNT in the ferret nerve–ganglion plexus, each containing two to more than thirty soma. Neurons, which have their soma in these ganglia, seem specialized for single transport because they provide

axons running both rostrally and caudally within the LNT (Coburn & Kalia, 1986). Microelectrode studies show the presence of evoked fast excitatory postsynaptic potentials (EPSPs) and spikes in only a proportion of the cell bodies in these ganglia (Baker *et al.* 1983; Cameron & Coburn, 1984). These neurons have been termed

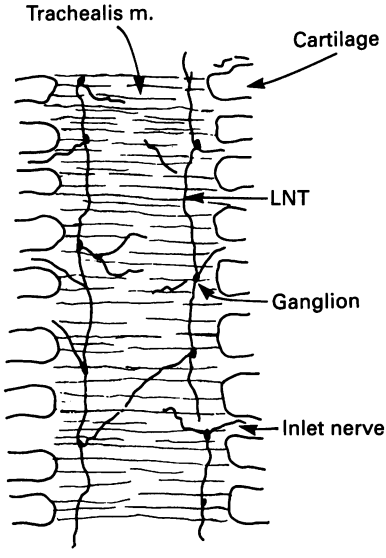

Fig. 1. Schematic representation of the anatomy of the ferret paratracheal nerve–ganglion plexus. The preparation of the nerve–ganglion plexus is described in the text.

airway after-hyperpolarization (AH) neurons because of their postaction potential after-hyperpolarization and some similarities to AH neurons in the myenteric plexus. It is not known whether these processes provide the motor innervation for smooth muscle cells in the airway wall. Additionally, there are large numbers of neurons (i.e. 'bridge neurons') which have their cell bodies separate from the LNT in the superficial plexus (Baker *et al.* 1986; Dey & Coburn, 1989).

Although there is descriptive information about several different pools of neurons in this plexus, there is little detailed information about the general organization of motor pathways innervating smooth muscle cells. The circuitry involved in motor innervation, whether the smooth muscle field innervated from one of the six to eight inlet nerves is demarcated, diffuse as in other autonomic ganglia (Lichtman, 1980; Hume & Purves, 1983) or whether input is convergent and overlapping (Hillarp, 1959), are all largely unexplored. We do not know of any previous study of the general organization of parasympathetic innervation of smooth muscle cells, and the characteristics of synapses and neurotransmitters involved.

Specific goals in the present study were: (*a*) to map the location of smooth muscle cells excited by neurons running in a single inlet nerve or neurons present at a single location on the LNT; (*b*) to determine characteristics of excitatory innervation occurring via these pathways; and, (*c*) to estimate the number of motor pathways involved in the excitatory innervation of single smooth muscle cells.

METHODS

Forty female ferrets, weighing 100–200 g, were used in this study. These animals were killed with inhaled carbon dioxide and exsanguinated. The chest was opened and the entire trachea removed and placed in chilled, gassed Krebs solution.

The preparation used has been fully described elsewhere (Cameron & Coburn, 1984). After opening the trachea along its ventral surface, tips of cartilages were removed to allow visualization of the nerve–ganglion plexus. The caudal part of the trachea, up to 3 cm from the carina, was pinned in the tissue chamber posterior surface up. The chamber was constantly perfused with Krebs solution at a flow rate of 6 ml min^{-1} and a temperature of 33–34 °C. The fascia over the dorsal surface of the trachealis membrane was removed to uncover the nerve–ganglionic plexus and smooth muscle. The plexus was visualized via bright field or dark field illumination at 20–40 × magnification.

The left longitudinal nerve trunk (LNT), or an inlet nerve entering the left portion of the plexus (see Fig. 1), was prepared for electrical stimulation. Nerves were elevated several millimetres away from the preparation and placed on bipolar stainless steel stimulating electrodes. Pulses were delivered with a Grass S44 stimulator via a stimulus isolation unit. Control stimulation performed without placing the nerve on the stimulating electrode did not produce electrical signals in smooth muscle or nerve cells.

Muscle membrane potentials were recorded using intracellular glass microelectrodes, filled with 1·5 M KCl, which had tip resistances of 40–60 MΩ. Signals were amplified with an electrometer (WPI Model 767, New Haven, CT, USA) and displayed on a storage oscilloscope. The criteria for successful penetrations were a rapid decrease in the membrane potential and a stable potential for 10 s. We selected muscle cells which had membrane potentials > 55 mV (about 95% of cells penetrated).

Excitatory junction potentials (EJPs) in smooth muscle cells were evoked by stimulating an inlet nerve or a LNT. In general, five to ten pulses (0·4–0·5 ms duration) were delivered over 100–200 ms. This evoked an EJP prior to onset of contraction. Three control experiments were performed to determine the effect of altering pulse frequency (12–50 Hz) on EJP amplitude, keeping the number of pulses constant (Fig. 3*B*). In two experiments we studied the effect of increasing the number of stimulating pulses over the same stimulation time period (Fig. 3*A*), increasing frequency from 12 to 60 Hz.

In the first experiments we used voltages 80–90% of maximum to minimize nerve damage. These experiments were performed at 30–50 Hz in order to obtain a 5–10 mV EJP. In later experiments we used 25 Hz stimulation at a voltage which was shown to give the maximal EJP amplitude.

We restricted our studies to smooth muscle cells located in three different areas (i.e. fields) of trachealis muscle; fields located transversely from the point of nerve stimulation, and fields located in the rostral or caudal direction from the point of nerve stimulation, 1 mm medial to the LNT. Locations of these fields are illustrated in subsequent figures. We penetrated at least four different smooth muscle cells in 1 mm^2 regions of each field.

In three experiments we used field stimulation to evoke EJPs. A stimulating bipolar electrode (the poles were separated by 0·3 mm) was placed on the surface of smooth muscle widely separated from the LNT or other visible nerve trunks. Smooth muscle cells located between the poles of this electrode were penetrated with microelectrodes. We used 0·1 ms pulses, 10–15 Hz and low voltage to evoke EJPs. This approach allowed us to study the myoneural junction; (however, EJPs may not purely reflect activation of postganglionic neurons located at the myoneural junction since there are cell bodies located in the area, even though stimulation was distant from the LNT).

Somal recordings from AH neurons present in ganglia associated with the LNT were also obtained. Nineteen AH neurons were studied. The criteria for successful penetration were the same as used for smooth muscle recordings. AH neurons were characterized by fast EPSPs and single action potentials evoked by LNT stimulation.

Solutions, drugs and statistics

Krebs solution contained (mM): Na^+, 137; Cl^-, 122; K^+, 5·9; Mg^{2+}, 1·2; PO_4^-, 1·2; Ca^{2+}, 2; glucose, 11·5. The solution was gassed with 5% CO_2–95% O_2. The pH of the solution was adjusted to 7·35–7·40 with HCO_3^- (giving a final [HCO_3^-] of 20–26 mM. Indomethacin (1 μM) was added to the

Krebs reservoir since others have shown that this retards run-down of nerve stimulation-evoked contractile responses in other airway muscle (DeJongeste, Mons, Bonta & Kerrebijn, 1987; Undem, Meyers, Barthlow & Weinreich, 1990). Drugs were added to a second reservoir of Krebs solution. After switching solutions it took 25–30 s for drugs to reach the tissue chamber and 3–5 min to achieve steady-state concentrations. Atropine, tetrodotoxin, indomethacin, hexamethonium, curare, capsaicin and propranolol were obtained from Sigma Chemical Chemical Co. Capsaicin was dissolved in 95% ethanol–water.

Data are given as means ± S.E.M. Statistical significance was determined using Student's t test for unpaired data. $P < 0.05$ was regarded as significant.

RESULTS

Basic characteristics of EJPs in tracheal smooth muscle cells

Representative EJPs are shown in Fig. 2. In common with other studies on airway smooth muscle, EJPs never evoked action potentials. The latency time (time from the beginning of stimulation to the EJP onset) varied in the range 50–200 ms, depending primarily on the distance of the penetrated muscle from the stimulating electrode. The duration of the EJP was a function of amplitude and varied between 0·3 and 1·5 s. EJPs developed monotonically. The initial upsweep and peak of the EJP occurred prior to onset of force development. There was a more prolonged repolarization; in about 30% of the measurements where the electrode was not displaced, the membrane remained slightly depolarized for several seconds or there was a small second phase of the potential (Fig. 2). The second phase of the membrane potential response was possibly related to displacement of the electrode due to muscle movement. Duplicate or triplicate stimulations using the same stimulus parameters produced EJPs in the same muscle cell whose amplitude varied less than ±10%.

EJPs summated as a function of the number of pulses delivered to inlet nerve or LNT (Fig. 3*A*). Three experiments were performed to determine effects of altering pulse frequency (12–50 Hz) on EJP amplitudes, keeping the number of pulses constant (Fig. 3*B*). In all of the fields studied single muscle cells followed frequencies as high as 25 Hz, but there was a fall in EJP amplitude at higher frequencies of stimulation. The coefficient of variation in EJP amplitudes measured in cells located within 1 mm^2 in the same field was < 11% (n = 3–8 cells per field). Atropine (10^{-7}–10^{-6} M) and TTX (1 $\mu g\ ml^{-1}$) completely abolished evoked EJPs under all experimental conditions. Propranolol (10^{-6} M) had no effect on EJP amplitude: control 8·4 ± 1·2 mV, propranolol 8·3 ± 0·5 mV (n = 8).

Location of muscle cells activated by stimulation of a single inlet nerve or region of a LNT

Amplitudes of EJPs recorded in smooth muscle cells located in the caudal direction from the point of nerve stimulation (Figs 4 and 5) decreased with increasing distances from the point of stimulation. EJPs were entirely absent at a distance of 4 mm (n = 8 experiments) from the location of the stimulated inlet nerve and 6·5 mm (n = 8 experiments) from the site of LNT stimulation. EJPs were evoked with nerve stimulation in fifty-three of sixty-four cells (83%) located within 4 mm of the point of stimulation (eight experiments). There was no frequency effect (between 25 and 30–50 Hz) on the size of the innervated field.

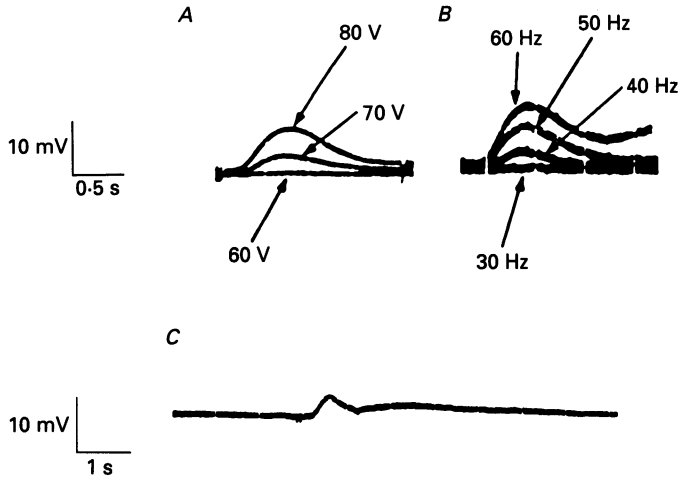

Fig. 2. Example EJPs recorded in airway smooth muscle cells. *A*, EJPs were evoked by inlet nerve stimulation at different voltages (40 Hz, 0·5 ms, for 200 ms); *B*, stimulation frequency was varied over a constant time period (200 ms, 0·5 ms duration, submaximal voltage). *C*, an evoked EJP which showed evidence of a biphasic response (25 Hz, for 200 ms, 0·4 ms, maximal voltage).

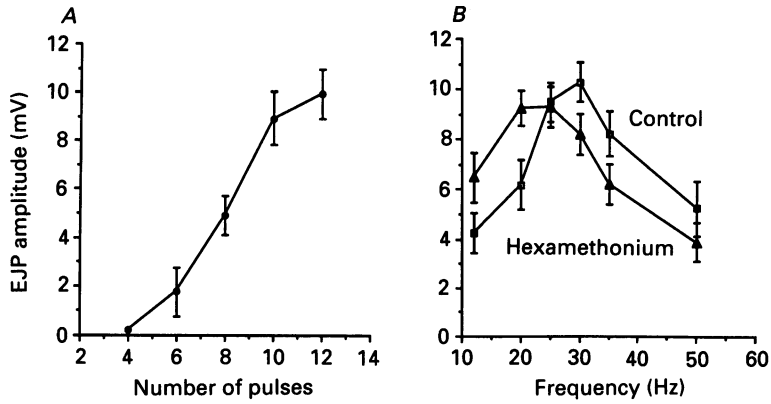

Fig. 3. *A*, relationship of number of stimulating pulses (LNT) to EJP amplitude. Measurements made in rostral fields in two preparations. Numbers of pulses were increased by increasing stimulation frequency from 12 to 60 Hz, over a constant time period (200 ms, 0·5 ms, maximum voltage). *B*, effect of stimulation frequency on EJP amplitude under control conditions and following hexamethonium. The total number of stimulating pulses was kept constant (five pulses, 0·5 ms, maximum voltage). Control and hexamethonium data were recorded from the same cell in each experiment. Hexamethonium data were obtained 15–30 min after this drug was added to the perfusing solution. Means ± S.E.M., $n = 3$ experiments.

EJPs were recorded in smooth muscle cells located in rostral fields more than 15 mm from the stimulating electrode (Figs 4 and 5). In contrast to data obtained on caudal fields, little or no decrement in EJP amplitude was seen with increasing distance from the stimulating electrode to a constant voltage of stimulation. There

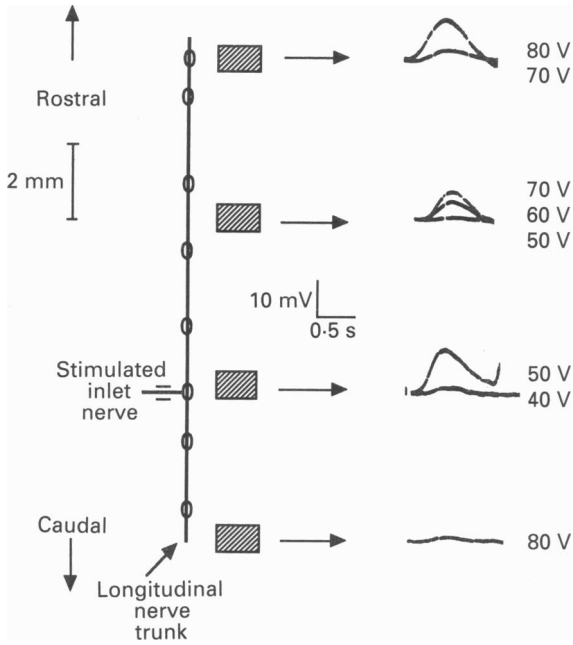

Fig. 4. Fields studied in the rostral and caudal direction from the stimulated inlet nerve. Example EJPs are also shown, taken at different stimulation voltages (40 Hz, 0·5 ms, 200 ms). Recording fields shown by hatched rectangles.

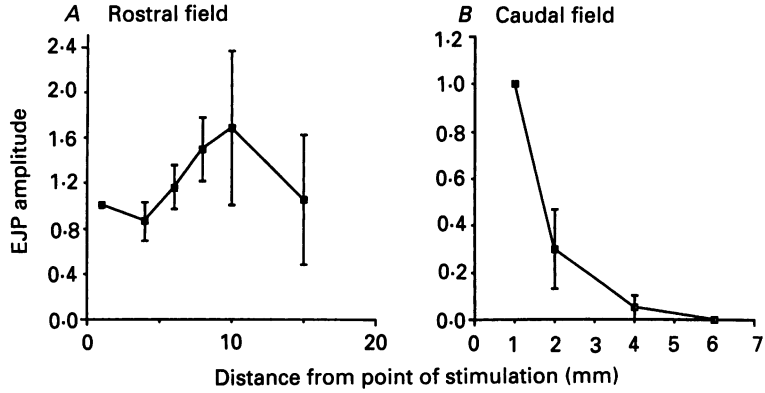

Fig. 5. Relative EJP amplitudes recorded in smooth muscle cells located in rostral and caudal fields progressively more distant from the stimulated inlet nerve. Stimulus parameters were the same in each individual experiment: 0·5 ms, 30–50 Hz, 150–200 ms. We normalized data obtained in each experiment to the EJP amplitude determined in cells located in fields 0·5 mm distant from the stimulating electrode. Means ± S.E.M., $n = 17$ experiments.

was no difference in data obtained comparing LNT and inlet nerve stimulation or changes in stimulation frequency. There was no demarcated area innervated by neurons in single inlet nerves. Of 103 cells studied in rostral fields, 86 showed EJPs (83%, seventeen experiments).

Innervated cells were seen in all transverse fields studied, including smooth muscle cells located on the opposite side of the preparation from the point of stimulation in close proximity to another inlet nerve. Of 153 cells studied in transverse fields, 146 showed EJPs. There was some decrement in EJP amplitude with distance but this was only significant at a distance of 3 mm from the site of nerve stimulation (Fig. 6).

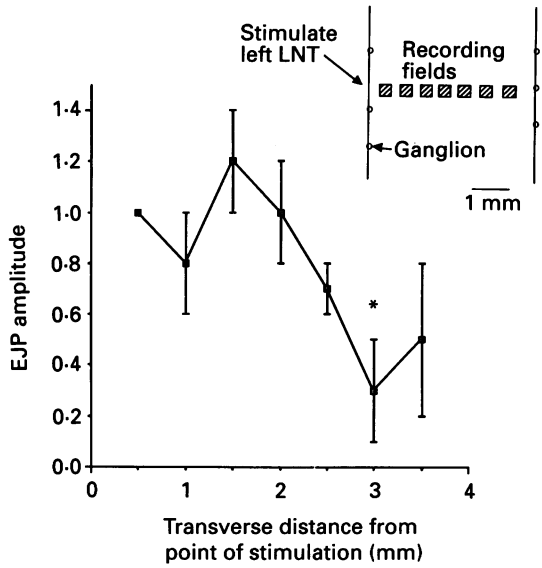

Fig. 6. EJP amplitudes *versus* distance in transverse fields. Data obtained and normalized as described in Fig. 5. Data were significantly decreased at 3 mm from the stimulating electrode, but we could not convincingly show decrement in EJP amplitudes with increasing distance from the other data points. The recording fields are shown in the inset.

Are smooth muscle cells innervated by motor pathways originating in different inlet nerves?

This was studied using smooth muscle cells located in two different fields: (a) a transverse field located midway between inlet nerves or LNT located on the left and on the right side of the preparation (eight cells); and (b) a rostral field 7 mm distant from the stimulated left inlet nerve or LNT (twelve cells). Inlet nerve or LNT stimulations on either the left or the right side of the preparation evoked EJPs in all muscle cells studied in either field. Figure 7 illustrates the data obtained in a transverse field in response to LNT stimulation.

Innervation by motor pathways originating in different neurons in the same LNT

Stimulating voltage was increased in multiple small steps and the number of step increases in EJP amplitude determined. Twelve muscle cells located in a field 7 mm rostral to the stimulation electrode were studied. Data are given in Fig. 8. In twelve cells studied, multiple step increases in voltage resulted in two step increases in EJP amplitude in four cells, three increases in EJP amplitude in seven cells and one increase in one cell.

Are signals carried in the LNT?

This was studied by transecting the LNT at various points and determining if this abolished or attenuated evoked EJPs. Data obtained in five experiments indicate that in both the caudal and rostral directions, motor pathways initially run in the

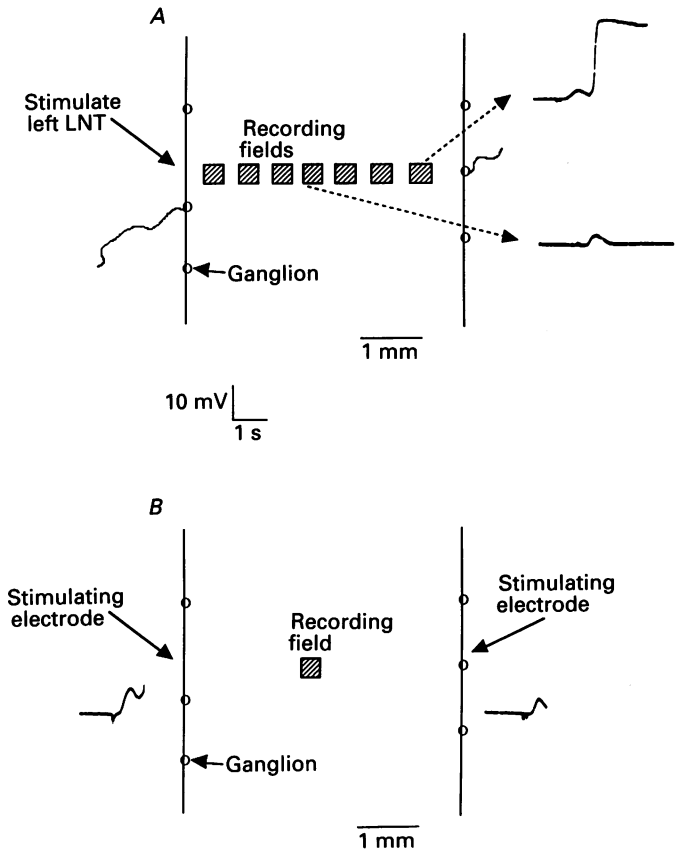

Fig. 7. *A*, example of results from an experiment illustrating that EJPs can be evoked in smooth muscle cells located across the preparation from the point of stimulation (25 Hz, 200 ms stimulation at maximum voltage). *B*, the finding that a single smooth muscle cell located in the middle of the preparation receives neural input from both LNTs (25 Hz, 200 ms stimulation at maximum voltage).

LNT. Motor signals were shown to exit from the LNT at a point (or level) perpendicular to the smooth muscle field studied.

Dependence of rostral and caudal pathways on nicotinic synapses

Rostral fields

Control EJPs were evoked in ten to fifteen different smooth muscle cells within the same field. Hexamethonium was perfused into the organ bath and the effect on evoked EJPs recorded in ten to fifteen other smooth muscle cells (located within

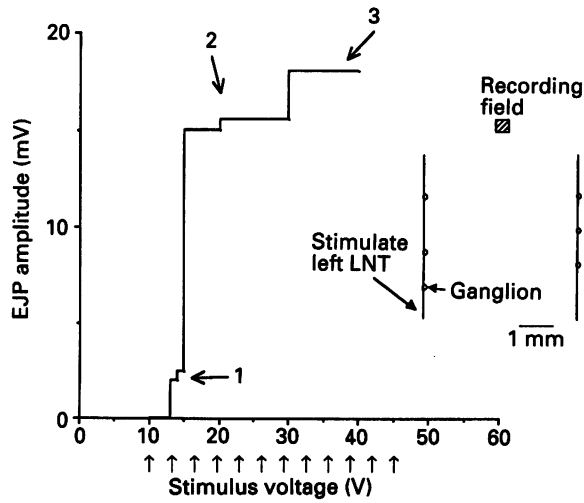

Fig. 8. The step-voltage approach to estimate the number of motor pathways that can be activated by stimulation of an LNT (50 Hz, 0·5 ms for 200 ms). The graph shows graded increases in stimulus voltage (arrows) and step increases in EJP amplitude (nos 1–3) recorded in a single smooth muscle cell. The recording field used in this experiment is shown in the inset.

TABLE 1. Effect of hexamethonium on EJP amplitude

Field	Unchanged*	Partly suppressed†	Abolished
Rostral			
< 2 mm	7 (11)	1 (11)	3 (11)
2–4 mm	6 (11)	1 (11)	4 (11)
4–10 mm	5 (11)	1 (8)	3 (8)
⩾ 15 mm	3 (7)	1 (7)	3 (7)
Caudal			
< 2 mm	4 (10)	0 (10)	6 (10)
2–4 mm	1 (10)	0 (10)	9 (10)
4–6 mm	0 (7)	0 (7)	7 (7)

Data summarize the number of experiments (given in parentheses) and the effect of hexamethonium on EJP amplitude at various locations (fields) either rostral to or caudal to the point of stimulation. Data from inlet nerve and LNT stimulations are combined. Responses to high voltages only were compared (see text for stimulus parameters). *Unchanged, < 25% inhibition in amplitude of EJPs; †partly suppressed, 25–75% inhibition in EJP amplitude.

0·5 mm of the cells penetrated for control recordings). Measurements were made starting 15 min after steady-state concentration (5×10^{-4}–10^{-3} M) was achieved in the organ bath fluid. The pattern of results varied in different rostral fields. In about half of the 1 mm^2 fields studied in the same preparation, evoked EJPs were entirely resistant to hexamethonium; in the remainder EJPs were entirely inhibited or partially inhibited. Table 1 lists these data. Whether a field contained cells which exhibited hexamethonium-resistant or -sensitive EJPs seemed independent of the distance from the stimulating electrode. Hexamethonium-resistant EJPs were always entirely inhibited by atropine (10^{-7}–10^{-6} M), as were hexamethonium-

sensitive EJPs (four experiments). Results with 5×10^{-5} M curare were identical to those for hexamethonium ($n = 4$).

In four experiments, evoked EJPs were recorded in the same rostral cell before and during hexamethonium treatment. In three of these cells evoked EJP amplitude was

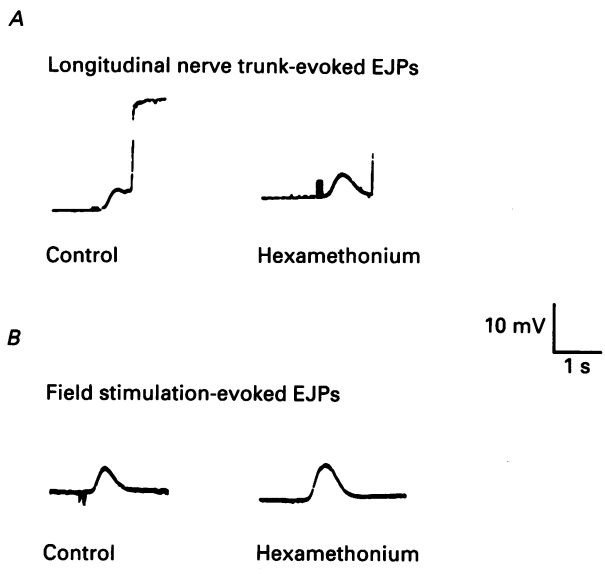

Fig. 9. Hexamethonium-resistant EJPs recorded in smooth muscle cells located in rostral fields. EJPs were evoked at 25 Hz, 0·5 ms, for 200 ms, maximal voltage. The rostral field was located 7 mm distant from the point of stimulation of the LNT (*A*). 'Control', recorded prior to addition of 10^{-3} M hexamethonium to the bathing solution. 'Hexamethonium', recorded 15–30 min after addition of hexamethonium. *B*, The lower panel shows example EJPs obtained from a muscle cell in the same field as shown in the upper panel, stimulated by placing the stimulating electrode (10 Hz, 0·1 ms pulses for 200 ms) on the surface of the smooth muscle close to the penetrated cell, i.e. 'field stimulation'.

not changed following hexamethonium (10^{-3} M); in the remaining cell EJP amplitude was suppressed. As expected, hexamethonium had no effect on EJPs evoked with field stimulation (control $5{\cdot}7 \pm 1{\cdot}2$ mV, hexamethonium $6{\cdot}1 \pm 1{\cdot}1$ mV, $n = 4$) (Fig. 9).

We compared the configurations of EJPs recorded prior to, and after addition of 10^{-3} M hexamethonium, recording from a field where hexamethonium-resistant EJPs were present. Two different indices were used: (a) peak amplitude divided by the time duration from EJP onset to peak amplitude; and (b) peak amplitude divided by total time duration of the entire EJP. Comparing twelve pairs, prior to hexamethonium, these were $29{\cdot}2 \pm 3{\cdot}4$ and $8{\cdot}7 \pm 2{\cdot}3$ mV s^{-1}, respectively. After hexamethonium these were $27{\cdot}1 \pm 3{\cdot}5$ and $9{\cdot}1 \pm 2{\cdot}4$ mV s^{-1}, respectively. Thus we were unable to detect any gross changes in EJP configuration resulting from hexamethonium treatment. In addition there were no effects of hexamethonium on the EJP latency time.

The frequency dependency for evoking hexamethonium-resistant EJPs is plotted in Fig. 3. Plots were not significantly different comparing control EJPs prior to

addition of hexamethonium and EJPs in the presence of hexamethonium. Hexamethonium-resistant EJPs were easily elicited using 0·1 ms pulses after an increase in stimulation voltage above threshold on the strength–duration curve. Hexamethonium-resistant pathways to individual rostral fields of smooth muscle cells were activated at both submaximal and maximum voltages of stimulation.

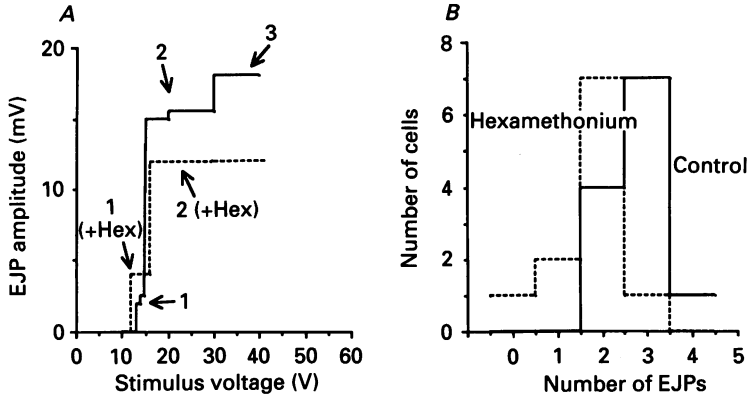

Fig. 10. Estimate of the numbers of pathways, present in LNT, which activate single smooth muscle cells in a rostral field. *A* shows the plot given in Fig. 8 (nos 1–3) and adds data from different cells in a nearby field (< 1 mm) obtained after circulating hexamethonium through the perfusion chamber (nos 1+2 (+Hex). *B*, summarizes all of the data obtained with twenty-three cells studied under hexamethonium and control conditions (50 Hz, 0·5 ms for 200 ms).

The multiple step increase in voltage approach showed that in eleven cells: one showed three step increases in EJP amplitude, seven showed two step increases, two showed one step increase in amplitude and in one cell there were no EJPs. Figure 10 shows these data and compares these data with results obtained in control experiments described above.

Amplitudes or configurations of hexamethonium-resistant EJPs were not changed following incubation of the preparation with 3–10 μM capsaicin for 30–60 min: mean EJP amplitude prior to capsaicin was $8{\cdot}2 \pm 1{\cdot}1$ mV and following capsaicin it was $7{\cdot}4 \pm 0{\cdot}8$ mV in a rostral field ($n = 12$).

We transected the LNT to determine if the hexamethonium-resistant pathway travels in this structure. The pathway was shown to initially run in the LNT but the signal exited from the LNT prior to reaching the level of the rostral field studied. In five experiments the motor nerve fibres travelled distances of 2, 5, 6, 5 and 7 mm in the LNT before exiting the trunk and activating muscle cells in fields 12–15 mm from the inlet nerve.

Table 2 summarizes the characteristics of hexamethonium-resistant rostral EJPs.

Caudal fields

Hexamethonium entirely abolished evoked EJPs in twelve of thirteen experiments in smooth muscle cells located in caudal fields, 2–4 mm from the stimulating electrode.

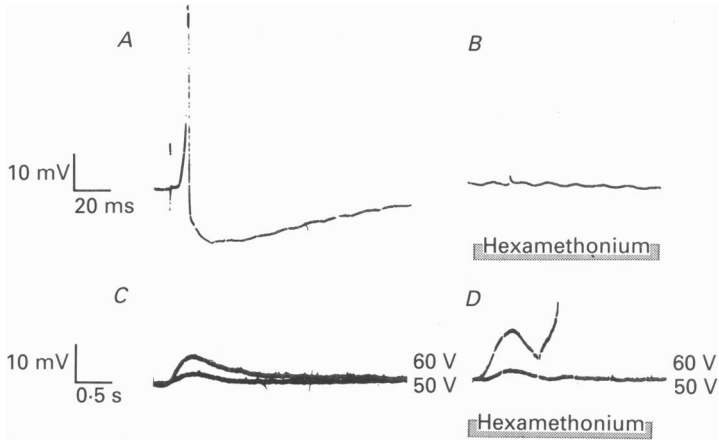

Fig. 11. Effect of hexamethonium on somal action potentials in an AH neuron (*A* and *B*) and nearby muscle cells (*C* and *D*), both located about 6 mm in the rostral direction from the point of inlet nerve stimulation. The neuron was stimulated using a single 0·5 ms pulse applied to an inlet nerve. The muscle stimulation used multiple pulses (50 Hz, 200 ms, two different voltages). As the hexamethonium effect developed, first the action potential was inhibited but small fast EPSPs could still be recorded. At 10 min (*B*) the EPSP was completely inhibited as well. Muscle EJPs were not inhibited. Vertical lines in *A* and *B* are stimulus artifacts.

TABLE 2. Characteristics of rostral hexamethonium-resistant motor pathway

(1) Runs initially in the LNT.
(2) EJP is entirely inhibited by atropine.
(3) Pathway does not include a synapse with LNT AH neurons.
(4) EJPs resistant to 10 μM capsaicin.
(5) Follows stimulation frequency to 25 Hz; activated by 0·1 ms pulses.
(6) Configuration of EJPs evoked via this pathway and their voltage dependency are not altered by hexamethonium treatment.
(7) More motoneurons, or groups of motoneurons, present in inlet nerves and LNT activate this pathway than activate hexamethonium-sensitive pathways.

Effects of hexamethonium on neurotransmission in AH neurons in the LNT

The resting membrane potential of these cells was $48{\cdot}3 \pm 11{\cdot}4$ mV ($n = 19$). These were not significantly different during circulation of hexamethonium through the bath. Figure 11 shows that hexamethonium abolished action potentials evoked by single LNT stimulation. This occurred in sixteen out of sixteen AH neurons studied. The same effect of hexamethonium occurred when we evoked fast EPSPs with single or with multiple (50 Hz) LNT stimulation. However, as shown in Fig. 11, EJPs evoked in smooth muscle cells were still present.

DISCUSSION

EJPs were evoked in 83–95% of the smooth muscle cells present in all of the fields studied. Our strategy was to evoke an EJP prior to the onset of muscle contraction which required high frequency, short duration stimulation. As in our previous study

(Coburn, 1984), single pulse stimulations of a LNT or inlet nerve failed to consistantly evoke an EJP (data not shown). This may be due to intermittency of transmitter release at low frequencies as seen in other nerve–muscle preparations (e.g. Brock & Cunnane, 1988), or another cause. Our findings argue against an organization of innervation where different inlet nerves activate motor pathways which supply smooth muscle cells located diffusely throughout trachealis muscle. The general pattern of motor innervation shown in the present study is one of overlapping innervation and convergence of motor pathways to single muscle cells or groups of muscle cells. Evidence includes: (a) stimulation of different inlet nerves or different LNTs resulted in evoked EJPs in the same smooth muscle cell; and (b) the fields of rostral and transverse smooth muscle cells innervated by one inlet nerve extended into areas in close approximation to other inlet nerves.

The concept of convergence of motor pathways to single smooth muscle cells was expanded to include different motoneurons which run in single regions of the LNT. The multiple step voltage-increase approach showed that recruitment of nerve fibres resulted in up to four step increases in EJP amplitudes in a given smooth muscle cell. The rationale for this approach was that recruited neurons, or groups of similar neurons in the LNT at increasing voltages of stimulation, increase the concentration of acetylcholine at the myoneural junction resulting in an increased EJP amplitude. Our finding suggests that there are four motor pathways, innervating smooth muscle cells, in one inlet nerve. In view of the reported scanty motor innervation to airway smooth muscle seen in other species (Baluk & Gabella, 1989; Gabella, 1989), our data may be best explained by the presence of electrically coupled smooth muscle cells and uniform and convergent innervation to groups of coupled cells. The order in which populations of motoneurons are recruited and the magnitude of individual step increases in EJP amplitude may depend on anatomical characteristics of fibre populations in the nerve trunks, and on intermittency of synaptic transmission and the amounts of transmitter released from activated circuits. This approach probably gives an underestimation of the actual number of motor pathways leading from a single inlet nerve to a single smooth muscle cell because of competition between circuitries activated at different voltages (Lichtman, 1977).

This study uncovered evidence that there is polarity in motor innervation leading from inlet nerves and LNTs to single smooth muscle cells. Data comparing single inlet nerve and LNT stimulations were practically identical suggesting that a similar population of motoneurons were stimulated with each approach. Although fibres passed in the LNT in both directions, smooth muscle cells were activated, in a field 1 mm from the LNT, at much further distances rostral than caudal to the point of stimulation. In a previous study no such limit in the caudal projection was observed (Coburn, 1984). The explanation for the different findings in this study and the present study was not determined. However, experimental conditions were different in the two studies; a likely explanation is that the previous studies used older ferrets.

The multiple step increase in voltage approach to quantitating the number of neurons, or groups of neurons, that activate single smooth muscle cells, gave data that suggest there are more hexamethonium-resistant pathways activated by LNT stimulation, than hexamethonium-sensitive pathways. This follows from the finding that most cells showed three step increases in EJP amplitude under conditions where

both pathways were operative (control data), and two step increases during hexamethonium. Since both pathways (hexamethonium sensitive and resistant) are activated over the same range of stimulating voltage, it is suggested there is no difference in distribution of sizes of neurons, or the distribution of myelinated *versus* non-myelinated neurons in the LNT, which activate the two different pathways.

The caudal motor pathways and the hexamethonium-sensitive rostral pathway can be explained by classical innervation: cholinergic preganglionic neurons synapse on postganglionic neurons via nicotinic synapses. The location of synapses could be on AH neurons in ganglia associated with the LNT. The nature of the rostral hexamethonium-resistant pathway has not been completely defined. There is evidence against a mechanism involving antidromic stimulation of afferent C fibres and release of tachykinins (as well as a classical mechanism) which activate smooth muscle. The capsaicin resistance of this pathway, the unchanged EJP configurations following hexamethonium, the voltage dependence and frequency dependence of this pathway, the complete inhibition of EJPs evoked by stimulation of this pathway by atropine, all argue against this postulate.

It is unlikely that non-nicotinic pathways in the ferret trachealis nerve–ganglion plexus could be explained by the presence in inlet nerves of postganglionic neurons that directly release ACh in proximity to smooth muscle cells. The magnitude and distribution of the hexamethonium-resistant EJPs and data obtained about motor pathways in other parasympathetic ganglia (Lichtman, 1980; Hume & Purves, 1983) argue against this. A few isolated soma were found in laryngeal nerves in the guinea-pig trachea (Baluk & Gabella, 1989), but we have been unable, using light microscopy, to identify soma in dissected inlet nerves in our preparation. The finding that neurotransmission, to all sixteen AH neurons tested, was inhibited by hexamethonium indicates that the concentration of this drug was high within the capsules of ganglia associated with the LNT; thus it is unlikely that the hexamethonium-resistant pathway is related to inadequate intracapsular concentration of this drug. Our data suggest that the LNT AH neurons are not involved in non-nicotinic circuitry to the smooth muscle in the rostral fields. Evoked EJPs were still present in approximately half the smooth muscle cells even when synaptic transmission at this ganglion cell was blocked by hexamethonium or curare.

Enkephalinase inhibitors result in augmentation of vagal stimulated-evoked contraction in the ferret trachealis muscle (Sekizawa, Tamaoki, Nadel & Borson, 1987). There is strong evidence of the presence of afferent C fibres (Lundberg, Brodin & Saria, 1983; Undem *et al.* 1990; Borson, 1991) in various airway smooth muscles which when stimulated antidromically release tachykinins which mediate contraction. Resistance of EJPs to capsaicin in the present study, does not exclude the importance of tachykinins released from intramural neurons during stimulation of inlet nerves or LNTs. Although capsaicin is known to deplete afferent C fibres of tachykinins, there is evidence that this drug does not deplete intrinsic motoneurons of these neuropeptides (Holzer, Rainer & Lembeck, 1980).

The present results are consistent with a postulate that there are peptidergic synapses in the LNT or in pathways involving bridge neurons or neurons leading to the final motoneuron which releases acetylcholine. Peptidergic neurons have been identified in close proximity to cell bodies of neurons which run in the LNT, using

electron microscopy (Cameron & Coburn, 1984). Non-nicotinic circuitry described here may involve peptidergic synapses with neurons outside the LNT ganglia. We found that the non-nicotinic circuitry extended from the LNT for considerable distances through the trachealis muscle (Fig. 12). Extraganglionic neurons in the

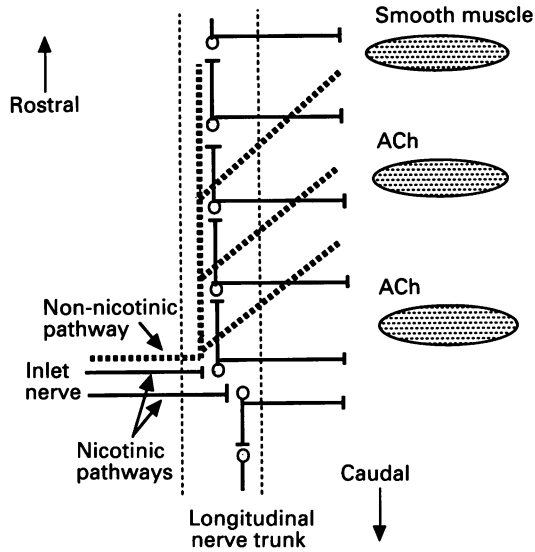

Fig. 12. Proposed pathways for innervation of trachealis muscle cells. Interrupted lines indicate non-nicotinic pathways, continuous lines indicate nicotinic pathways. The proposed circuitry incorporates the major findings of this study: nicotinic and non-nicotinic pathways transversing the rostral LNT and leaving this trunk to innervate smooth muscle; a single nicotinic pathway projecting in the caudal direction. The proposed pathway suggests multiple nicotinic synapses in different ganglia in the LNT. The final neurotransmitter at the myoneural junction is acetylcholine.

plexi overlying the trachealis muscle have been shown to contain multiple neuropeptides (Dey & Coburn, 1989). Substance P activates neurons in peripheral airway ganglia in the guinea-pig bronchus (Undem *et al.* 1990), increased the amplitude of evoked EJPs in three out of six runs in our preparation (H. W. Mitchell & R. F. Coburn, unpublished observations), and has been shown to have an excitatory effect on neurotransmission in other parasympathetic ganglia (Kawatami, Whitney, Booth & De Groat, 1989).

Our data have defined the general organization of innervation, and allow a first estimate of the number of motor pathways that can excite single smooth muscle cells. There is a polarity in the innervation of smooth muscle cells located in different directions from the point of inlet nerve stimulation. A rostral hexamethonium-resistant motor pathway has been defined. Figure 12 summarizes schematically the various motor pathways identified in this study.

This work was funded in part by grant R 37 H137498-04 from the National Heart, Blood and Lung Institute, NIH, Bethesda, MD, USA. H.W.M. thanks the Perkins Foundation for support.

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