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RELAXANT INNERVATION OF THE GUINEA-PIG TRACHEALIS: DEMONSTRATION OF CAPSAICIN-SENSITIVE AND -INSENSITIVE VAGAL PATHWAYS

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SUMMARY

1. The guinea-pig trachea was isolated with its extrinsic innervation intact and placed in a water-jacketed dissecting dish containing warmed, oxygenated Krebs solution. The trachea was not separated from the oesophagus. Two adjacent cartilage rings of the rostral portion of the trachea were cut open opposite the trachealis and prepared for isometric tension measurements.

2. Following the addition of atropine and contraction of the trachealis with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), stimulation of the cervical sympathetic trunks elicited relaxations that were abolished by propranolol or hexamethonium. Stimulation of the vagus nerves caudal to the nodose ganglia also elicited relaxations. These vagally mediated relaxations were unaffected by propranolol but were abolished by hexamethonium or by cutting the recurrent laryngeal nerves.

3. After cutting the vagi caudal to the nodose ganglia, stimulation of the vagi rostral to the nodose ganglia elicited relaxations of the trachealis that were not significantly affected by either propranolol or hexamethonium but were abolished by cutting the superior laryngeal nerves. Stimulation of right vagi which had undergone supranodose vagotomy 14 days prior to experimentation was without effect on the smooth muscle of the guinea-pig trachea while the response to stimulation of the left vagus was unchanged.

4. Acute capsaicin desensitization abolished relaxations of the guinea-pig trachealis elicited by stimulation of the vagal fibres carried by the superior laryngeal nerves. In contrast, capsaicin desensitization only modestly inhibited relaxations elicited by stimulation of the preganglionic parasympathetic fibres carried by the recurrent laryngeal nerves and had no effect on sympathetic nerve-induced relaxations.

5. Removing the oesophagus selectively abolished relaxations elicited by stimulation of both vagal pathways of non-adrenergic relaxant innervation. Non-adrenergic relaxations of the trachealis elicited by electrical field stimulation were unaffected by removing the oesophagus. Oesophagus removal also had no effect on the parasympathetic–cholinergic contractile innervation or the sympathetic relaxant innervation of the trachealis.

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6. The results indicate that the guinea-pig trachealis receives non-adrenergic relaxant innervation from both parasympathetic and capsaicin-sensitive vagal pathways. The results also suggest that the neurones mediating non-adrenergic relaxations of the trachea are sensitive to oesophagus removal. The observation that oesophagus removal abolishes parasympathetic relaxations of the trachealis while having no effect on parasympathetic contractions supports the hypothesis that the guinea-pig trachealis receives excitatory and inhibitory innervation from distinct vagal parasympathetic pathways.

INTRODUCTION

Guinea-pig airway smooth muscle receives both adrenergic and non-adrenergic relaxant innervation (Coburn & Tomita, 1973; Coleman & Levy, 1974). The former can be elicited by stimulation of sympathetic nerves, whereas the non-adrenergic innervation is thought to arise from the vagus nerves (Burden, Parkes & Gardiner, 1971; Chesrown, Venugopalan, Gold & Drazen, 1980; Yip, Palombini & Coburn, 1981).

The neurotransmitter mediating non-adrenergic bronchodilatation has not been identified. Several putative neurotransmitters have been implicated as mediators of non-adrenergic relaxations induced by electrical field stimulation (EFS) of isolated guinea-pig trachealis, including vasoactive intestinal peptide and/or peptide histidine isoleucine (Matsuzaki, Hamasaki & Said, 1980; Ellis & Farmer, 1989), adenosine (Coleman & Levy, 1974; Satchell, 1984) and nitric oxide (Li & Rand, 1991; Tucker, Brave, Charalambous, Hobbs & Gibson, 1991).

One disadvantage to using the isolated muscle strip to study airway smooth muscle innervation is that the various neural systems present in the tissue cannot be independently stimulated. Thus, the smooth muscle responses elicited by EFS are the combined effects of neurotransmitters released from adrenergic (Foster, 1964; Coburn & Tomita, 1973), cholinergic (Carlyle, 1964) and capsaicin-sensitive (Lundberg & Saria, 1982) nerve endings. Non-neural effects of electrical field stimulation on airway smooth muscle preparations have also been described (Ullman, Ciabattoni, Svedmyr, Skoogh & Löfdahl, 1991). These drawbacks of EFS have led to the development of isolated, innervated airway smooth muscle preparations with which responses can be elicited by stimulation of the intact extrinsic nerves (Blackman & McCaig, 1983; Widmarck & Waldeck, 1986; Undem, Myers, Barthlow & Weinreich, 1990).

Surprisingly, vagally mediated, non-adrenergic relaxations have been difficult to elicit *in vitro*, even in preparations that have intact contractile innervation (Blackman & McCaig, 1983; Widmarck & Waldeck, 1986; Undem *et al.* 1990; Pendry & Maclagan, 1991). Non-adrenergic relaxations can, however, be elicited in these same preparations by EFS (Blackman & McCaig, 1983; Undem *et al.* 1990). Thus, isolating guinea-pig airways for *in vitro* study may selectively disrupt the extrinsic pathways of the non-adrenergic relaxant innervation.

We have developed a preparation with which the extrinsic innervation of an isolated segment of the rostral portion of the guinea-pig trachea can be studied functionally. The advantage of using the rostral portion of the trachealis is that the parasympathetic, sympathetic and afferent innervation of this region of the tissue

are carried by anatomically distinct extrinsic nerves (Smith & Satchell, 1985; Baluk & Gabella, 1989), making it possible to study the efferent properties of each system independently. Consistent with the literature, we found that the sympathetic innervation of this part of the trachealis is derived from the superior cervical ganglia with preganglionic fibres carried by the cervical sympathetic trunks. Relaxations induced by sympathetic nerve stimulation are inhibited by propranolol and thus appear to be mediated by β-adrenoceptors. Vagus nerve stimulation uncovered two distinct pathways of non-adrenergic relaxant innervation: a hexamethonium-sensitive (parasympathetic) relaxant innervation with preganglionic fibres carried by the recurrent laryngeal nerves and a previously undescribed hexamethonium-insensitive relaxant innervation composed of capsaicin-sensitive vagal neurones carried by the superior laryngeal nerves. Finally, we present data consistent with the hypothesis that vagally mediated non-adrenergic relaxations of the guinea-pig trachea are completely dependent on the integrity of neurones that are in some way associated with, perhaps even intrinsic to, the oesophagus.

METHODS

Male Hartley guinea-pigs (200–400 g, Harlan Sprague–Dawley Inc., Indianopolis, IN, USA) were asphyxiated by inhalation of 100% CO_2 and exsanguinated. The spinal column (C1 to T5), larynx, trachea, mainstem bronchi, carotid arteries, jugular veins, oesophagus and all associated tissues were removed and placed in a dissecting dish containing oxygenated Krebs solution of the following composition (mM): NaCl, 118; KCl, 5·4; NaH_2PO_4, 1; $MgSO_4$, 1·2; $CaCl_2$, 1·9; $NaHCO_3$, 25; dextrose, 11·1. The vagus nerves and the cervical sympathetic trunks were separated from the surrounding tissues by blunt dissection. Care was taken not to disrupt the recurrent laryngeal or the superior laryngeal nerves branching from the vagi or the fibres emanating from the intact sympathetic ganglia. The carotid arteries and jugular veins were removed. Once the major vessels and all other extraneous tissues had been removed, the airways and their extrinsic innervation (including the stellate, midcervical and superior cervical sympathetic ganglia) were freed from the thoracic cavity and spinal column and pinned dorsal-side down to the floor of a water-jacketed dissecting dish continuously overfilled (20 ml min^{-1}) with warmed (37 °C), oxygenated Krebs solution.

Inconsistent results were obtained when the trachea was pulled free from the oesophagus. We reasoned that removing the oesophagus disrupted the dense neural network seen under low-power magnification in the space between the trachea and the oesophagus. Consequently, unless otherwise stated, the oesophagus was not removed.

When the blunt dissection was complete, the rostral portion of the trachea was cut open longitudinally opposite the trachealis. Two adjacent tracheal rings (rings 6 and 7 caudal to the larynx) were prepared for isometric tension measurements. One end of the rings was pinned to the bottom of the dissecting dish while the other end was attached to a force transducer (Grass model FT03C, Grass Instruments, Quincy, MA, USA). The optimal baseline tension (1·5 g) was set and continuously adjusted during a 60 min equilibration period. Changes in isometric tension were recorded on a Grass polygraph (model 79E). An illustration of the preparation is shown in Fig. 1.

Nerve stimulation

The extrinsic nerves were stimulated with suction electrodes (World Precision Instruments, Sarasota, FL, USA) attached to a Grass stimulator (Model S44) which delivered square pulses of current at supramaximal stimulus intensities (see Fig. 1 for details of electrode placement). The threshold voltage for stimulation of the various extrinsic nerves using this electrode arrangement and a 1 ms pulse duration was between 2 and 25 V, with half-maximal voltages ranging from 10 to 50 V. The voltage used (150 V) was about twice that required for maximal stimulation. The left and right nerves were stimulated simultaneously for 10 s at frequencies ranging from a single pulse (0·1 Hz) to 32 Hz. Based on preliminary studies of the frequency–response characteristics of the

various responses elicited, optimal stimulation frequencies were used for the characterization of the neuronal pathways.

In several experiments, the tracheae were stimulated with EFS by passing current between chlorided silver-chloride electrodes placed on either side of the preparations. Preliminary experiments demonstrated that square pulses of 50 V at a pulse duration of 1 ms were optimal for evoking tetrodotoxin-sensitive responses.

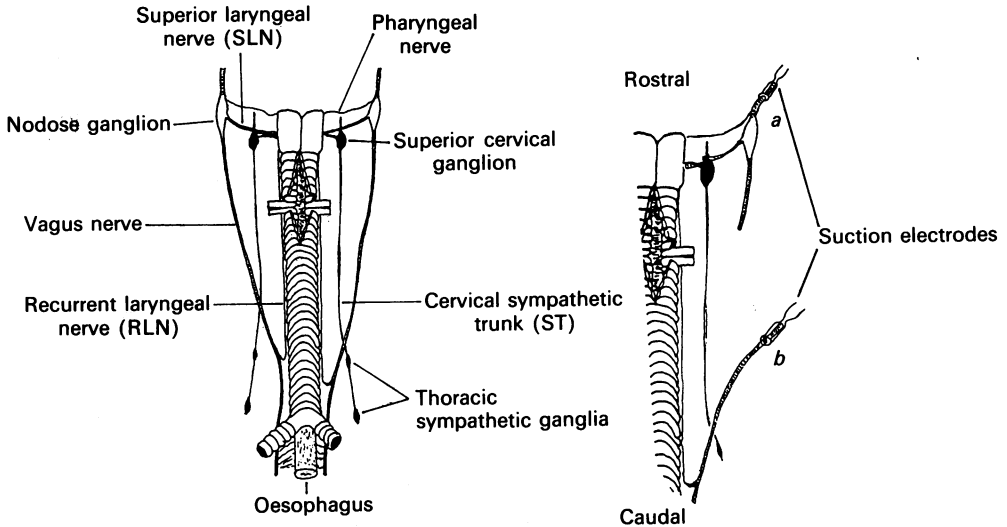

Fig. 1. *A*, an illustration of the preparation used to study the extrinsic innervation of the guinea-pig trachealis. Isometric tension of the opened tracheal rings (rings 6 and 7 caudal to the larynx) was measured by connecting the rings to a force transducer. Unless stated otherwise, the oesophagus was left intact. The right and left sympathetic trunks were cut and their rostral ends stimulated with suction electrodes. The recurrent laryngeal nerves were stimulated (with suction electrodes) 5 mm rostral to their origin in the vagus nerves. *B*, an illustration of the placement of electrodes for selective stimulation of the rostral (*a*; superior laryngeal and pharyngeal nerves intact) or caudal (*b*, recurrent laryngeal nerves intact) vagal pathways. Electrodes were placed on both right and left vagus nerves.

Extrinsic nerve-mediated relaxations

At the end of the 60 min equilibration period, the integrity of the vagal innervation of each preparation was tested. The right, then left and then both vagus nerves were stimulated (16 Hz, 10 s train duration) rostral to the nodose ganglia. Preparations that did not respond to each of the aforementioned nerve stimulation protocols (less than 2 % of all preparations) were not used in these studies. Subsequent to stimulation, the vagi were cut about 5 mm caudal to the nodose ganglia. Unless otherwise stated, tissues were then treated with atropine (1 μM) and contracted to 90 % of their maximum by adding 1 μM $PGF_{2\alpha}$ to the perfusate. The contraction elicited by $PGF_{2\alpha}$ remained stable throughout the course of the experiment. Relaxations in response to extrinsic nerve stimulation were elicited after the $PGF_{2\alpha}$-induced contraction reached equilibrium. At the end of each experiment a maximal relaxation of the smooth muscle was induced by adding 0·1 mM papaverine to the buffer. All nerve-mediated relaxations are expressed as a percentage of this maximum response.

Pharmacological studies

The effects of various pharmacological agents on the nerve-mediated relaxations were assessed using a paired experimental design. Extrinsic nerve-mediated relaxations were elicited before and

immediately after a 25 min incubation in drug-containing buffer. Time controls for each response measured in which no drug was present during the 25 min period between stimulations were carried out in parallel. The rationale for the 25 min incubation period was based on results from preliminary time course experiments. The various drugs used, at the concentrations chosen, were maximally effective after a 20 min incubation period. Unless otherwise stated, a maximal stimulation frequency (24 Hz) was used for these studies.

Capsaicin desensitization

To assess the role of capsaicin-sensitive neurones in the nerve-mediated responses, the effect of capsaicin desensitization was tested. Capsaicin desensitization was carried out *in vitro* by exposing tissues to buffer containing 3 μM capsaicin for 60 min at the outset of an experiment followed by a 30 min incubation in capsaicin-free buffer. Nerve-mediated responses of capsaicin-treated tissues were compared to nerve-mediated responses of tissues exposed to buffer containing the vehicle for capsaicin (ethanol). We have previously demonstrated that this treatment selectively blocks neurally mediated non-cholinergic contractions of the guinea-pig isolated bronchus (Undem *et al.* 1990) and trachea (Ellis & Undem, 1990).

Effect of removing the oesophagus on nerve mediated responses

The effects of oesophagus removal on nerve-mediated responses were studied systematically by comparing responses of preparations having an oesophagus to responses in preparations in which the oesophagus was partially (leaving only that portion of the oesophagus contiguous with the rostral half of the trachea intact) or completely removed. In other preparations, rather than removing a portion of the oesophagus, blunt dissection in the space between the trachea and oesophagus was carried out. Small haemostats were carefully guided into the space between the oesophagus and tracheal rings 6 and 7 caudal to the larynx, taking great care not to disrupt the recurrent nerves. Once the tip of the haemostats had passed through the space entirely, the haemostats were carefully opened, disrupting the tissue occupying the space between the oesophagus and tracheal rings 3–9 caudal to the larynx.

In a separate set of experiments, vagally mediated responses of the oesophagus were measured. The caudal end of the oesophagus was cannulated and continuously perfused (20 ml min^{-1}) with warmed (37 °C) Krebs solution. A pressure transducer (Statham Model P23, Gould Inc., Oxnard, CA, USA) attached to a side port of the cannula measured the oesophageal inflow pressure. The buffer flowed out of the oesophagus through a hole made in its dorsal side at the level of the larynx. The buffer flowing through the oesophageal lumen ultimately filled and overflowed from the dissecting dish. Vagally mediated changes in oesophageal inflow pressure and either isometric tension of the trachealis or intraluminal pressure of the trachea were measured simultaneously. Intraluminal pressure of the trachea was measured using a technique comparable to that used by Blackman & McCaig (1983). During intervals between nerve stimulations, however, the tracheal lumen was continuously perfused (20 ml min^{-1}) with Krebs solution stored separate from that perfusing the oesophagus. This design allowed selective drug delivery to the tracheal lumen.

Supranodose vagotomy

The technique used to administer unilateral supranodose vagotomy (as well as all other techniques described in this manuscript) was approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions. Guinea-pigs were anaesthetized for 1–2 h with an intramuscular injection of ketamine (50 mg kg^{-1}) and xylazine (2·5 mg kg^{-1}) dissolved in 0·9% saline. The surgery lasted approximately 15 min. Incisions were made right of centre on the animal's ventral surfaces proximal to the right nodose ganglia. The right vagi rostral to the nodose ganglia were dissected free from the surrounding tissues and cut rostral to the nodose ganglia and the superior laryngeal branches of the vagus nerves. The incisions ($<$ 2 cm) were sutured shut and the animals were then left to recover. Two weeks after the surgery, the tracheae were prepared as described above. The right and left vagi were stimulated singly, above and below the nodose ganglia both before and after the addition of atropine and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to the buffer. Responses of tissues from vagotomized animals were compared to responses of tissues from animals receiving sham vagotomies.

Pretreatments

We attempted to circumvent any influence endogenously formed prostanoids might have on our preparation (Undem *et al.* 1990) by adding 3 μM indomethacin to the buffer. In addition, we added 1 μM prazosin, the α_1-adrenoceptor-selective antagonist, to block sympathetic nerve-mediated contractions of this preparation (B. J. Canning & B. J. Undem, unpublished observation). All studies of vagally mediated relaxations were carried out in the presence of 1 μM propranolol.

Statistical analysis

The statistical significance of the effects of ganglionic blocking drugs on vagally mediated relaxations were assessed by two factor analysis of variance (reductions in vagally mediated relaxations as a function of time and/or as a function of ganglionic blockade). The effects of capsaicin, manipulating the oesophagus and supranodose vagotomy on nerve-mediated responses were assessed by Student's unpaired t test. P values of less than 0·05 were considered statistically significant.

Drugs

Atropine sulphate, 8-methyl-*N*-vanillyl-6-nonenamide (capsaicin, 98% pure), ketamine hydrochloride, xylazine, indomethacin, hexamethonium chloride, prazosin hydrochloride, DL-propranolol hydrochloride, papaverine hydrochloride, $PGF_{2\alpha}$, lidocaine, succinylcholine chloride and tetrodotoxin were purchased from Sigma (St Louis, MO, USA). Trimethaphan camsylate was acquired from Roche Laboratories (Nutley, NJ, USA). Atropine (10 mM), hexamethonium (1 M), succinylcholine (1 M), tetrodotoxin (1 mM), papaverine (50 mM) and propranolol (10 M) were dissolved in distilled water. Indomethacin (30 mM), capsaicin (10 mM), lidocaine (1 M) and $PGF_{2\alpha}$ (10 mM) were dissolved in 100% ethanol. Prazosin (10 mM) was dissolved in 50% ethanol. Trimethaphan (84 mM) was dissolved in 0·013% sodium acetate. Ketamine (50 mg ml^{-1}) and xylazine (2·5 mg ml^{-1}) were dissolved together in 0·9% saline.

RESULTS

Sympathetic nerve induced relaxations

Subsequent to the addition of 1 μM atropine and contraction of the tissues with 1 μM $PGF_{2\alpha}$, stimulation of the rostral ends of the cut cervical sympathetic trunks elicited frequency-dependent relaxations (Fig. 2). Stimulation of the caudal ends of the cut cervical sympathetic trunks was without effect ($n = 3$). The frequency at which a maximum response was elicited by a 10 s stimulation was 24 Hz. In four representative experiments, the average time required for the relaxations elicited by sympathetic nerve stimulation (24 Hz), 10 s stimuli) to return from their peak level of relaxation ($60·2 \pm 11·6$% of the maximum relaxation elicited by 0·1 mM papaverine) to 50% of the prestimulation tone was $21·8 \pm 1·4$ s (see Fig. 3). Propranolol (1 μM) or hexamethonium (1 mM) abolished sympathetic nerve-induced relaxations. The relaxations elicited by stimulation of the cervical sympathetic trunks at an optimal frequency (24 Hz, 10 s stimuli) averaged $62·9 \pm 15·8$% and 0% of the maximum response before and after propranolol, respectively ($n = 3$). Likewise, the relaxations averaged $69·6 \pm 7·2$% and 0% of the maximum response, before and after hexamethonium, respectively ($n = 4$). Figure 2 illustrates the effect of propranolol and hexamethonium on the complete frequency–response curve for relaxations elicited by stimulation of the cervical sympathetic trunks. Addition of 0·3 mM trimethaphan also substantially inhibited relaxations elicited by stimulation (24 Hz, 10 s stimuli) of the sympathetic trunks ($81·6 \pm 6·6$ and $14·2 \pm 6·1$% of the maximum response before and after trimethaphan, respectively; $n = 5$).

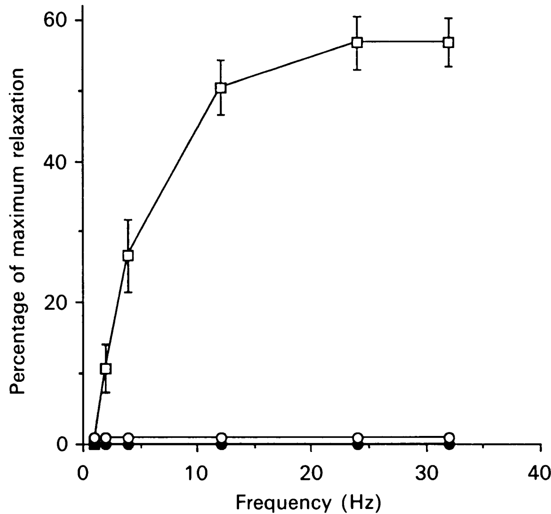

Fig. 2. Frequency-dependent relaxations of the isolated, innervated guinea-pig trachea elicited by stimulation of the rostral ends of the cut cervical sympathetic trunks. Responses are presented as a mean ± S.E.M percentage of the maximum relaxation elicited by 0·1 mM papaverine added at the end of each experiment. Frequency–response curves were generated by stimulating the nerves at 6 min intervals with a 10 s train of pulses at supramaximal stimulus intensities of progressively higher frequencies. Curves were generated before and immediately after a 25 min interval during which tissues were exposed to no drugs (□, n = 6), 1 μM propranolol (○, $n = 3$) or 1 mM hexamethonium (●, $n = 2$). The peak magnitude of relaxations elicited by stimulation (24 Hz, 10 s stimuli) of the sympathetic trunks prior to the administration of propranolol (63 ± 16) or hexamethonium (67 ± 8) was not different from that elicited in the untreated tissues (61 ± 4; $P > 0·1$).

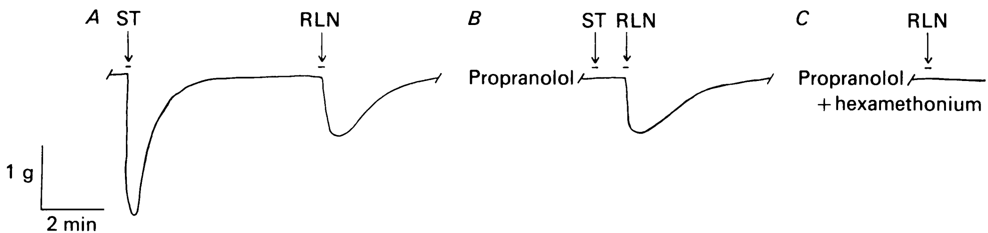

Fig. 3. Representative trace of relaxations of the isolated, innervated guinea-pig trachea elicited by stimulation of the rostral end of the cut cervical sympathetic trunks (ST) or the recurrent laryngeal nerves (RLN). Nerves were stimulated (24 Hz, 10 s train, supramaximal stimulus intensity) in the absence of drugs (*A*) and then 25 min subsequent to the addition of propranolol (1 μM; *B*) and propranolol and hexamethonium (0·1 mM; *C*). Atropine (1 μM) was present throughout. Arrows denote the onset of bilateral nerve stimulation.

To determine if any postganglionic fibres arising from thoracic sympathetic ganglia reached the rostral trachealis via the recurrent laryngeal nerves, the right and left recurrent nerves were stimulated 5 mm rostral to their origin in the vagus nerves. The relaxations elicited by stimulation (24 Hz, 10 s stimuli) of the recurrent

laryngeal nerves averaged $23 \cdot 6 \pm 2 \cdot 0$ % of the maximum response, lasted for a longer period of time than the relaxations elicited by sympathetic nerve stimulation ($81 \cdot 5 \pm 5 \cdot 3$ s to return from peak relaxation to 50 % of prestimulation level) and were unaffected by propranolol ($23 \cdot 6 \pm 2 \cdot 0$ and $20 \cdot 4 \pm 1 \cdot 4$ % of the maximum response before and after propranolol, respectively). The fibres of the recurrent laryngeal nerves mediating relaxation appeared to be a preganglionic inasmuch as the relaxations were abolished by 0·1 mM hexamethonium ($n = 4$). A representative trace of relaxations elicited by stimulation of the cervical sympathetic and recurrent laryngeal nerves is illustrated in Fig. 3.

Vagally mediated responses

Contractions

Stimulation of the right or left vagus nerve rostral to the nodose ganglia (16 Hz, 10 s stimuli) elicited robust contractions of the trachealis in all of the preparations studied. A single pulse delivered to the vagi also elicited contractions of the trachealis. The contractions elicited by stimulation of the vagi were rapid in both onset and decay, with approximately 80 % of the contraction occurring during the first 6 s of stimulation and returning to within 80 % of the original baseline tone in less than 15 s subsequent to the cessation of stimulation. These contractions were blocked by 1 μM atropine. In five of fifty tissues, the rapid cholinergic contractions were followed by slowly developing, long-lasting (1–5 min) contractions. These vagally mediated second-phase contractions were reminiscent of the capsaicin-sensitive, tachykinin-mediated contractions of the guinea-pig trachea elicited by EFS (Ellis & Undem, 1990).

In fifty preparations, the vagus nerves were cut caudal to the nodose ganglia and stimulated either rostral to the ganglia (i.e. superior laryngeal and pharyngeal pathways intact) or at the peripheral cut end (recurrent laryngeal pathways intact; see Fig. 1). These two pathways will be referred to as the rostral vagal pathways and the caudal vagal pathways, respectively. In all fifty experiments, rapid, atropine-sensitive contractions were elicited by stimulation of the caudal vagal pathways. By contrast, comparable rapidly developing contractions were elicited by stimulating the rostral vagal pathways in only seven of the preparations. Stimulation of the rostral vagal pathways resulted in the slower-second phase contractions in twelve of the fifty preparations.

In another set of experiments the tissues were treated with 3 μM capsaicin to render the tachykinin-containing afferent fibres of the tissue inactive (see Methods). Addition of capsaicin (60 min application, 30 min washout) induced contractions of the trachealis ($1 \cdot 4 \pm 0 \cdot 2$ g in thirteen representative experiments) which peaked rapidly and gradually returned to baseline 30–40 min subsequent to administration. The magnitude of vagally mediated contractions (elicited (16 Hz, 10 s stimuli) after the 30 min wash-out period) was slightly enhanced by capsaicin pretreatment. The contractions averaged $1 \cdot 4 \pm 0 \cdot 2$ g ($n = 10$) and $1 \cdot 8 \pm 0 \cdot 1$ g ($n = 13$) in vehicle control and capsaicin-pretreated tissues, respectively ($P < 0 \cdot 01$). In only two of forty-five preparations pretreated with capsaicin were second-phase contractions elicited by stimulation of the rostral vagal pathways.

Vagally mediated non-adrenergic relaxations

In the presence of atropine (1 μM) and propranolol (1 μM), stimulation of either the caudal or rostral vagal pathways elicited frequency-dependent relaxations of the guinea-pig trachealis in all control tissues studied ($n = 21$). The frequency–response relationship of the vagally mediated relaxations was not different between the two pathways. The frequency at which maximum relaxations were elicited by a 10 s stimulation of the vagi both rostral and caudal to the nodose ganglia was 24 Hz and the minimum frequency to elicit a relaxation was 4 Hz ($n = 6$).

The magnitude and kinetics of the relaxations elicited by stimulation of the caudal vagal pathways were virtually identical to those elicited by stimulation of the recurrent laryngeal nerves (see above). The peak magnitude of the response to a 10 s stimulation at 24 Hz averaged 36.2 ± 3.2% of the maximum response elicited by papaverine ($n = 21$). In thirteen representative experiments, the time required for the tension to return from the peak relaxation to 50% of the prestimulation level averaged 91.6 ± 6.0 s. In five of five experiments, cutting the recurrent laryngeal nerves at tracheal ring 16 caudal to the larynx abolished relaxations elicited by stimulating the caudal vagal pathways.

The kinetics of the relaxations elicited by stimulating (24 Hz, 10 s stimuli) the rostral vagal pathways were similar to those elicited by stimulation of the caudal vagal pathways. In twelve representative experiments, the average time required to return from the peak relaxations to 50% of the prestimulation tone was 74.8 ± 6.3 s. The magnitude of the relaxations elicited by stimulation (24 Hz, 10 s stimuli) of the rostral vagal pathways were smaller, on average, than those elicited by stimulation of the other extrinsic nerves (15.7 ± 1.8% of the maximum response; $n = 21$). In three experiments the rostral vagal pathways were stimulated at 24 Hz continuously until a maximum (steady-state) relaxation was obtained. These relaxations reached a steady state by about 90 s that averaged 22.0 ± 4.5% of the maximum response. In three of three experiments, cutting the superior laryngeal nerves abolished these relaxations. Cutting the pharyngeal nerves had no effect on these relaxations ($n = 3$).

Effect of ganglionic blocking drugs on vagally mediated relaxations

In the next series of experiments, relaxations were elicited by stimulation of either the caudal or rostral vagal pathways before and 25 min after the addition of ganglionic blocking drugs. Time control experiments carried out in parallel to the pharmacological studies revealed that the magnitude of relaxations elicited by stimulation of either the caudal or rostral vagal pathways of the vagus nerves were reduced subsequent to a 25 min period during which no drugs were added ($P < 0.05$). Accordingly, reductions in vagally mediated relaxations subsequent to ganglionic blockade were assessed as a function of both time and drug. Hexamethonium (0·1 mM) or trimethaphan (50 μM) virtually abolished relaxations elicited by stimulation of the caudal vagal pathways. By contrast, the apparent reductions in the magnitude of relaxations elicited by stimulation of the rostral vagal pathways subsequent to ganglionic blockade could not be distinguished from the effects of elapsed time alone ($P > 0.1$). Furthermore, in four additional experiments, a 10-fold

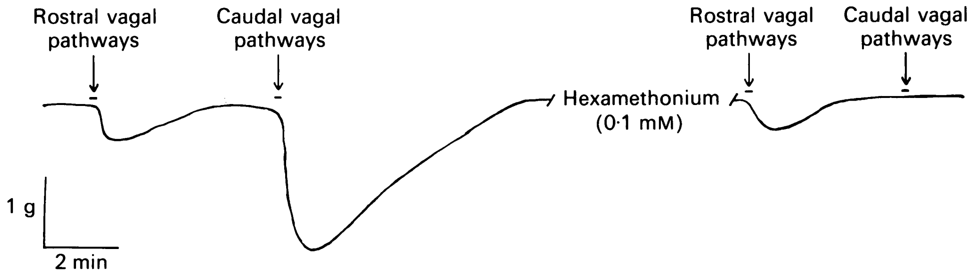

Fig. 4. Representative trace of relaxations of the isolated, innervated guinea-pig trachea elicited by stimulation of the rostral or caudal pathways of the vagus nerves (see Fig. 1 for electrode placement allowing independent stimulation of the rostral and caudal vagal pathways) before and immediately after a 25 min incubation in the presence of 0·1 mM hexamethonium. The vagi were stimulated bilaterally for 10 s at 24 Hz with supramaximal stimulus intensities. Propranolol (1 μM) and atropine (1 μM) were present throughout the experiment.

TABLE 1. Effect of autonomic ganglion blockers on vagally mediated relaxations of the guinea-pig trachea

	Caudal pathways of the vagus nerves			Rostral pathways of the vagus nerves		
Drug administered	Control	Treated	n	Control	Treated	n
Time control	32·3 ± 7·7	25·6 ± 7·6	5	18·6 ± 5·0	16·2 ± 5·0	5
Hexamethonium						
0·1 mM	44·8 ± 5·5	3·6 ± 1·4*	6	12·8 ± 2·2	6·9 ± 0·5	6
1 mM	—†	—		21·6 ± 2·9	15·1 ± 2·6	4
Trimethaphan						
50 μM	39·3 ± 5·9	1·6 ± 0·8*	6	15·9 ± 3·6	10·6 ± 2·2	6

Relaxations of the isolated, innervated guinea-pig trachea were elicited by vagus nerve stimulation (24 Hz, 10 s train duration, supramaximal stimulus intensity) before (control) and after (treated) drug addition. Time control experiments in which the vagi were stimulated before and after a 25 min period during which no drugs were added were carried out in parallel. Vagi were cut caudal to the nodose and stimulated bilaterally, rostral (vagal fibres carried by the superior laryngeal nerves) or caudal (vagal fibres carried by the recurrent laryngeal nerves) to the nodose ganglia (see Fig. 1). Results are expressed as mean (±S.E.M.) percentage of the maximum relaxation elicited by 0·1 mM papaverine. Experiments carried out in the presence of 1 μM atropine and 1 μM propranolol. Asterisk denotes a statistically significant reduction in nerve-mediated relaxations due to drug ($P < 0·05$). Statistical comparison of group means were assessed as a function of time and drug by two-way ANOVA. † Experiment not done.

increase in the concentration of hexamethonium (1 mM) was without effect on relaxations elicited by stimulation of the rostral vagal pathways. The effects of ganglionic blockade on vagally mediated relaxations are illustrated in Fig. 4 and summarized in Table 1.

Effect of capsaicin desensitization on vagally mediated relaxations

Capsaicin pretreatment virtually abolished relaxations elicited by stimulating (24 Hz, 10 s stimuli) the rostral vagal pathways. The relaxations averaged 15·0 ± 1·8 ($n = 10$) and 1·4 ± 1·0 % ($n = 13$) of the maximum response in vehicle control and

capsaicin-pretreated tissues, respectively ($P < 0{\cdot}01$). By contrast, in these same experiments, relaxations elicited by stimulation (24 Hz, 10 s stimuli) of the caudal vagal pathways were only about 30% smaller following capsaicin pretreatment ($51{\cdot}5 \pm 5{\cdot}6$% and $34{\cdot}5 \pm 3{\cdot}7$% of the maximum response in vehicle control and

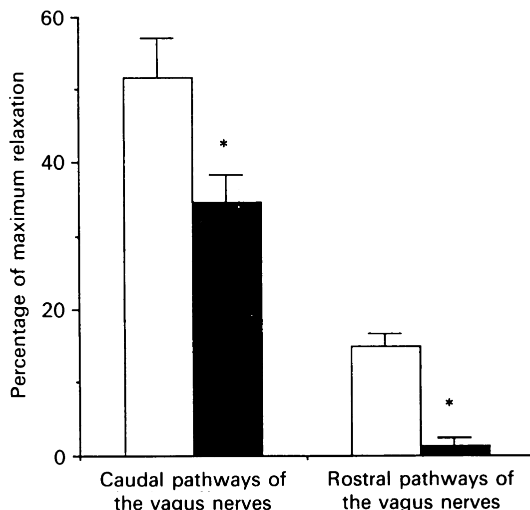

Fig. 5. Vagally mediated relaxations of isolated, innervated guinea-pig tracheae exposed to vehicle (open bars; $n = 10$) or 3 μM capsaicin (filled bars; $n = 13$). Electrodes were placed so that the caudal and rostral vagal pathways could be independently stimulated (see Fig. 1). Both vagal pathways were stimulated (24 Hz, 10 s stimuli), bilaterally with supramaximal stimulus intensities. Data are presented as a percentage of the maximum relaxation elicited by 0·1 mM papaverine. Bars represent the means ± S.E.M. Asterisk denotes a statistically significant reduction in nerve-mediated relaxations relative to vehicle control ($P < 0{\cdot}01$, Student's unpaired t test).

capsaicin-pretreated tissues, respectively ($P < 0{\cdot}01$). Relaxations elicited by stimulation (24 Hz, 10 s stimuli) of the sympathetic trunks averaged $58{\cdot}0 \pm 8{\cdot}3$% and $69{\cdot}7 \pm 6{\cdot}6$% in vehicle control ($n = 4$) and capsaicin-pretreated tissues ($n = 6$, respectively ($P > 0{\cdot}1$). The effects of capsaicin on vagally mediated relaxations are illustrated in Fig. 5. There was no significant difference between the average magnitude of the contractions elicited by 1 μM $PGF_{2\alpha}$ in vehicle control ($2{\cdot}5 \pm 0{\cdot}2$ g, $n = 10$) and capsaicin-pretreated tissues ($2{\cdot}7 \pm 0{\cdot}2$ g, $n = 13$), respectively ($P > 0{\cdot}5$).

Effect of removing the oesophagus on nerve-mediated responses

With the aid of a dissecting microscope, the oesophagus was removed from the preparation. Care was taken to avoid disrupting the recurrent laryngeal nerves. Removing the oesophagus abolished all vagally mediated relaxations (Fig. 6, Table 2). Removing only that portion of the oesophagus contiguous with the caudal half of the trachea was without effect on vagally mediated relaxations (Table 2).

In another series of experiments we left the oesophagus intact but disrupted, with

blunt dissection (see Methods), any nerve fibres that might connect the oesophagus and tracheal rings 3–9 caudal to the larynx. This also abolished relaxations elicited by stimulation of the caudal vagal pathways (Table 2).

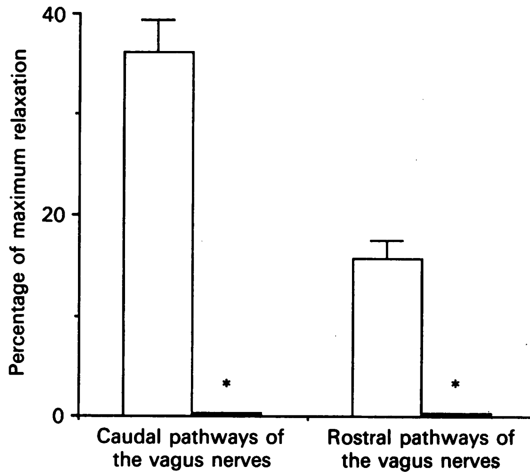

Fig. 6. Vagally mediated relaxations of isolated, innervated guinea-pig tracheal preparations with the oesophagus intact (open bars; $n = 21$) or removed (filled bars; $n = 10$). The electrodes were placed so that the caudal and rostral vagal pathways could be independently stimulation (see Fig. 1). Both vagal pathways were stimulated (24 Hz, 10 s stimuli) bilaterally with supramaximal stimulus intensities. The data are expressed as a percentage of the maximum relaxation elicited by 0·1 mM papaverine. Bars represent the means ± S.E.M. Asterisk denotes a statistically significant reduction in nerve-mediated relaxations relative to control (oesophagus intact) ($P < 0{\cdot}01$, Students unpaired t test). These data are also summarized in Table 2.

The effects of removing the oesophagus or blunt dissection between the oesophagus and trachea were selective for the vagally mediated relaxations. These manipulations had no effect on either the non-adrenergic relaxations elicited by EFS (induced (32 Hz, 10 s stimuli) in the presence of propranolol) or on the adrenergic relaxations elicited by sympathetic nerve stimulation (24 Hz, 10 s stimuli; see Table 2). Removing the oesophagus was also without effect on the peak magnitude of contractions elicited by stimulation (16 Hz, 10 s stimuli) of the vagi (2·4 ± 0·4 g and 2·1 ± 0·3 g in the presence or absence of the oesophagus, respectively). Paranthetically, however, removing the oesophagus did increase the probability of observing the long-lasting, capsaicin-sensitive contractions that can be evoked by stimulation of the rostral vagal pathways (see above). These contractions were observed in fifteen out of eighteen preparations lacking an oesophagus while, as mentioned above, they were observed in only twelve of fifty preparations with the oesophagus intact. The peak magnitude of these second phase contractions averaged 21 ± 5 % of the vagally mediated cholinergic contractions.

We next addressed the possibility that the oesophagus was mediating tracheal relaxation by mechanically influencing the measurement of isometric tension. The oesophageal muscle response was evaluated by perfusing the lumen of the oesophagus

TABLE 2. Effect of surgical manipulations of the oesophagus *in vitro* on nerve-mediated relaxations of the isolated guinea-pig trachea

	Adrenergic relaxations	Non-adrenergic relaxations[a]		
Treatment	Sympathetic trunks	Caudal pathways of the vagus nerves	Rostral pathways of the vagus nerves	Electrical field stimulation
Cumulative controls[b]	66·4 ± 3·7 (24)	36·2 ± 3·2 (21)	15·7 ± 1·8 (21)	78·4 ± 4·9 (4)
Caudal two-thirds of oesophagus removed[c]	59·3 ± 4·5 (6)	25·0 ± 5·1 (6)	18·8 ± 2·1 (6)	81·2 ± 4·2 (6)
Oesophagus completely removed	62·6 ± 7·4 (4)	0 ± 0[d] (10)	0 ± 0[d] (10)	73·3 ± 7·3 (4)
Blunt dissection between trachea and oesophagus[e]	47·4 ± 12·6 (6)	0 ± 0[d] (6)	—[f]	80·7 ± 2·0 (6)

Relaxations of the isolated, innervated guinea-pig trachea were elicited by nerve stimulation (24 Hz, 10 s train duration, supramaximal stimulus intensity) or electrical field stimulation (32 Hz, 10 s train duration, 50 V, 1 ms pulse duration). Vagi were cut caudal to the nodose ganglia and stimulated bilaterally, rostral (vagal fibres carried by superior laryngeal nerves) or caudal (vagal fibres carried by recurrent laryngeal nerves) to the nodose ganglia. Results are expressed as a percentage of the maximum relaxation elicited by 0·1 mM papaverine. The number of experiments (n) is given in parentheses.

[a] Experiments carried out in the presence of atropine (1 μM) and propranolol (1 μM).
[b] Mean ± S.E.M. of all control (oesophagus intact, no capsaicin pretreatment) responses measured.
[c] See Methods. Oesophagus contiguous with the rostral half of the trachea intact.
[d] Statistically significant difference between value and control (oesophagus intact) value ($P < 0·01$, Student's unpaired t test).
[e] See Methods. Experiments carried out subsequent to capsaicin desensitization.
[f] Experiment not done.

and measuring changes in the oesophageal inflow pressure. In twenty-three experiments, stimulation of the right or left vagus nerve induced an increase in oesophageal inflow pressure. The onset of the oesophageal response was immediate upon initiating the stimulus, reaching a steady state within 2 s of stimulation then rapidly returning to baseline within seconds of ending the stimulus. By contrast, the tracheal relaxations elicited by stimulation (24 Hz, 10 s stimuli) of either the rostral or caudal vagal pathways were delayed in onset, beginning about 3–5 s after initiating the stimulus (i.e. after the oesophageal response had reached a steady state). The relaxations of the trachealis continued to increase in magnitude with a linear slope even after the oesophageal response had returned to baseline (Fig. 7). In short, no response of the oesophagus temporally coincided with the slowly developing, long-lasting vagally mediated relaxations of the trachealis. In two experiments we attempted to selectively block the vagally mediated oesophageal responses with atropine and succinylcholine. Addition of 1 μM atropine and 0·1 mM succinylcholine dramatically inhibited vagally mediated responses of the oesophagus while isometric relaxations of the trachea persisted (Fig. 7).

Measurement of the relaxations of the trachea was dependent on induced tone. In the absence of $PGF_{2\alpha}$, stimulation of the vagus nerves caused cholinergic contractions but not relaxations. $PGF_{2\alpha}$ had no effect, however, on oesophageal inflow pressure ($n = 23$). In a separate set of experiments, $PGF_{2\alpha}$ failed to elicit isometric contraction of the circular or longitudinal smooth muscle of the guinea-pig oesophagus, both of which contracted in response to 1 μM carbachol ($n = 2$).

We next designed an experiment in which the muscle responses of the trachea and oesophagus could be monitored simultaneously while drugs could be selectively administered to the trachea. The lumens of the trachea and oesophagus were perfused with separate buffer solutions and the muscle responses of both tissues were quantified as changes in pressure. As with all other experimental designs, the preparation was submerged in water-jacketed dish continuously overfilled with warmed, oxygenated Krebs solution (see Methods). Stimulation (24 Hz, 10 s stimuli) of the intact vagi rostral to the nodose ganglia (i.e. both caudal and rostral vagal pathways stimulated) increased intratracheal pressure $27·3 \pm 1·2$ cmH_2O and oesophageal inflow pressure $38·7 \pm 4·8$ cmH_2O ($n = 16$; Fig. 8). Addition of $PGF_{2\alpha}$ increased the intraluminal pressure of the trachea $21·2 \pm 1·2$ cmH_2O while, as previously stated, having no effect on the inflow pressure of the oesophagus ($n = 12$). When the $PGF_{2\alpha}$-induced increase in intratracheal pressure reached equilibrium, stimulation (24 Hz, 10 s stimuli) of the intact vagus nerves elicited a biphasic change in intratracheal pressure: an initial increase followed by a decrease in intraluminal pressure. In four experiments, addition of atropine to the bathing solution abolished the initial increase in intratracheal pressure while the decrease in intratracheal pressure persisted ($51·3 \pm 11·2$ and $43·0 \pm 13·4$ % reversal of $PGF_{2\alpha}$-induced increases in intratracheal pressure before and after addition of 1 μM atropine, respectively).

Adding lidocaine (100 μM) to the solutions bathing both the trachea and the oesophagus abolished all vagally mediated responses of the tissues ($n = 3$). By contrast, in three experiments, selective administration of lidocaine to the tracheal perfusate inhibited by more than 80 % the decreases in intratracheal pressure elicited by stimulation (24 Hz, 10 s stimuli) of the vagus nerves ($17·0 \pm 1·6$ and

$3{\cdot}0 \pm 1{\cdot}5$ cmH_2O, before and after lidocaine, respectively) but had no effect on the vagally mediated increases in oesophageal inflow pressure ($36{\cdot}7 \pm 19{\cdot}0$ and $34{\cdot}3 \pm 17{\cdot}6$ cmH_2O before and after lidocaine, respectively). Addition of lidocaine to the tracheal perfusate was also without effect on $PGF_{2\alpha}$-induced increases in

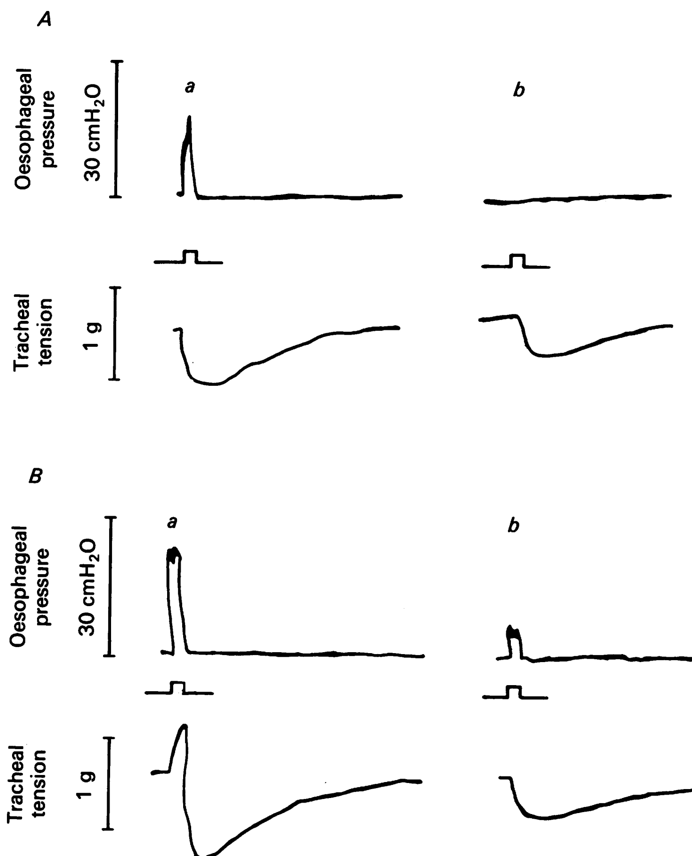

Fig. 7. Representative tracing of the simultaneous measurement of vagally mediated changes in oesophageal inflow pressure and isometric tension of the rostral trachealis. Effect of stimulating the rostral vagal pathways (*A*) or the caudal vagal pathways (*B*) on oesophageal inflow pressure and isometric tension of the trachealis (see Fig. 1 for electrode placement used to independently stimulate the caudal and rostral vagal pathways). Propranolol (1 μM) and $PGF_{2\alpha}$ (1 μM) were present throughout the experiment. In each experiment the vagi were stimulated in the absence (*a*) and in the presence (*b*) of 1 μM atropine and 0·1 mM succinylcholine. The raised bars between the tracheal and oesophageal traces denote the 10 s vagus nerve stimulation (24 Hz, supramaximal stimulus intensities). This tracing is representative of two similar experiments.

intratracheal pressure ($17{\cdot}0 \pm 3{\cdot}1$ and $15{\cdot}7 \pm 0{\cdot}7$ cmH_2O before and after intratracheal administration of 0·5 mM lidocaine, respectively). The vagally mediated tracheal relaxations could also be selectively abolished by adding 1 μM tetrodotoxin to the solution perfusing the trachea (Fig. 8).

Effect of supranodose vagotomy on vagally mediated responses

Supranodose vagotomy of the right vagus nerve 14 days prior to experimentation abolished all responses (both contractions and relaxations) to right vagus nerve stimulation while the responses to left vagus nerve stimulation persisted ($n = 3$).

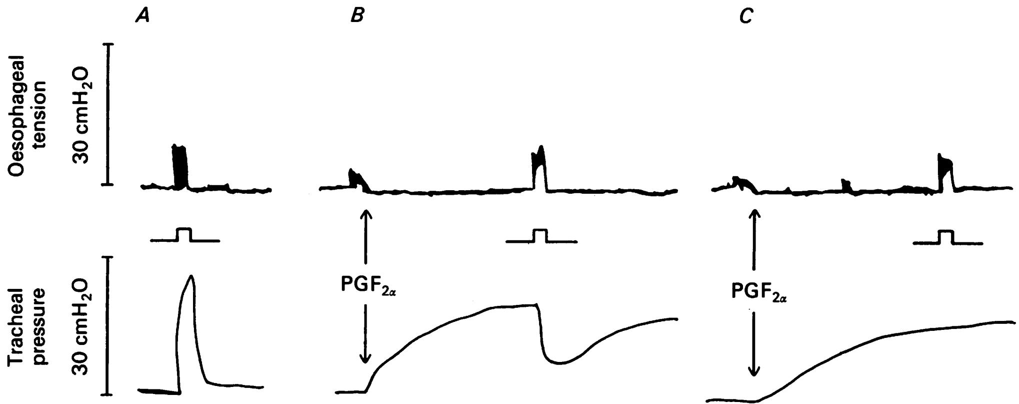

Fig. 8. Representative tracing of the simultaneous measurement of vagally mediated changes in oesophageal inflow pressure and intratracheal pressure in the guinea-pig isolated trachea–oesophagus preparation. Intact vagus nerves were stimulated bilaterally rostral to the nodose ganglia for 10 s at 24 Hz with supramaximal stimulus intensities. The 10 s periods of nerve stimulation are denoted by the raised bars between the tracheal and oesophageal traces. In *A*, the vagus nerves were stimulated in the absence of $PGF_{2\alpha}$-induced tone (propranolol (1 μM) was present throughout the experiments). Note the rapid increase in both the oesophageal and tracheal pressure. The oesophageal inflow pressure oscillated at the same frequency (24 Hz) as vagus nerve stimulation, hence the thickening of the pressure trace during stimulation. Prior to nerve stimulation in *B* the tissues were treated with 1 μM atropine and 1 μM $PGF_{2\alpha}$. The $PGF_{2\alpha}$ had no effect on oesophageal pressure but increased the tracheal pressure. Vagus nerve stimulation caused a non-adrenergic dilatation (decrease in pressure) of the trachea that was temporally unrelated to the rapid increase in oesophageal pressure. The $PGF_{2\alpha}$ was then removed from the perfusate and for the next 30 min, the tracheal lumen was selectively perfused with buffer containing tetrodotoxin (1 μM). At the end of this 30 min incubation, $PGF_{2\alpha}$ was added back to the perfusing solution in *C*. Notice that selectively perfusing the tracheal lumen with tetrodotoxin had no effect on the vagally mediated increase in oesophageal pressure but abolished the tracheal responses. This tracing is representative of two similar experiments.

DISCUSSION

The principal finding of this paper is that the guinea-pig trachealis receives relaxant innervation from at least three distinct extrinsic sources. In addition to adrenergic relaxant innervation derived from the cervical sympathetic ganglia, the rostral portion of the guinea-pig trachea receives non-adrenergic relaxant innervation from two vagal pathways: a hexamethonium-sensitive relaxant innervation with preganglionic fibres carried by the recurrent laryngeal nerves as well as a previously undescribed non-adrenergic relaxant innervation comprised of capsaicin-sensitive vagal neurones carried by the superior laryngeal nerves. The data presented also suggest that vagally mediated relaxations, but not contractions, are dependent on neurones associated with the oesophagus.

Sympathetic relaxant innervation

Functional and histochemical analyses have demonstrated the existence of sympathetic adrenergic innervation of guinea-pig airway smooth muscle (Foster, 1964; Burden *et al.* 1971; O'Donnell & Saar, 1973; Coburn & Tomita, 1973; Chesrown *et al.* 1980; Yip *et al.* 1981; Blackman & McCaig, 1983; Smith & Satchell, 1985). The present study demonstrates that the rostral portion of the guinea-pig trachealis receives its adrenergic innervation from postganglionic fibres that emanate from the superior cervical ganglia and merge with the rostral portion of the recurrent laryngeal nerves prior to innervating the trachea. The data also suggest that the rostral trachealis is not innervated by postganglionic adrenergic fibres carried by the caudal portions of the recurrent laryngeal nerves. These data are consistent with the histochemical studies of Smith & Satchell (1985).

We found no evidence of non-adrenergic relaxations in response to cervical sympathetic trunk stimulation. This is consistent with the observations of Chesrown and co-workers (guinea-pig) as well as those of Diamond & O'Donnell (cat) (Chesrown *et al.* 1980; Diamond & O'Donnell, 1980).

Parasympathetic relaxant innervation

Stimulation of the vagi caudal to the nodose ganglia elicited relaxations that were insensitive to the β-adrenoceptor antagonist propranolol but virtually abolished by the autonomic ganglion blockers hexamethonium and trimethaphan. This is consistent with the observations of Yip and co-workers (1981) who demonstrated that vagally mediated dilatation of the tracheal pouch *in situ* is abolished by hexamethonium. Thus, the rostral trachea receives parasympathetic contractile (cholinergic) and relaxant (non-adrenergic) innervation.

The vast majority of the preganglionic parasympathetic neurones projecting to the rostral trachealis reach the trachea by way of the recurrent laryngeal branches of the vagus nerves. In addition to the data discussed above, this hypothesis is based on the observation that the ganglionic blocking drugs had no significant effect on the relaxations elicited by stimulation of the rostral vagal pathways and that stimulation of the rostral vagal pathways did not elicit contractions with parasympathetic (cholinergic) characteristics in forty-three of fifty preparations studied.

Capsaicin-sensitive relaxant innervation

The relaxations elicited by stimulating the rostral vagal pathways of the vagus nerves were not inhibited by the ganglionic blocking drugs. These long-lasting non-adrenergic relaxations could not be elicited by stimulation of the cervical sympathetic trunks and thus are unlikely to be mediated by postganglionic sympathetic neurones. That the concentrations of trimethaphan and hexamethonium used were inadequate to block ganglionic transmission seems unlikely, since a 10-fold increase in the concentration of hexamethonium also had no effect on these relaxations. Furthermore, the concentrations of trimethaphan and hexamethonium used abolished relaxations elicited by stimulation of the caudal vagal pathways.

It is likely that the relaxations elicited by stimulation of the rostral vagal pathways are induced by antidromic stimulation of afferent fibres. This would

explain the resistance of these relaxations to ganglionic blockade as well as their sensitivity to capsaicin pretreatment. Capsaicin is a neurotoxin relatively selective for small afferent fibres (see Maggi, 1991). Indeed, our capsaicin protocol did not inhibit the cholinergic contractions or the adrenergic relaxations of our preparation and only marginally inhibited the non-adrenergic relaxations elicited by stimulation of the preganglionic parasympathetic fibres carried by the recurrent laryngeal nerves.

The nerve fibres of the rostral vagal pathways could reach the trachealis by way of the superior laryngeal nerves, pharyngeal nerves, or both (see Fig. 1). Our experiments in which the responses were studied before and after cutting these nerves demonstrate that the relaxant fibres of the rostral vagal pathways reach the trachealis via the superior laryngeal nerves. The finding that supranodose vagotomy 14 days prior to experimentation abolished parasympathetic and capsaicin-sensitive contractions and relaxations elicited by vagus nerve stimulation is suggestive evidence that the somata of the preganglionic parasympathetic fibres and the capsaicin-sensitive vagal fibres mediating these responses reside in the brain or in the jugular ganglia.

Under the appropriate conditions (oesophagus removed, no capsaicin pretreatment), stimulation of the rostral vagal pathways elicited contractions which were slow in onset and long in duration. Capsaicin pretreatment inhibited these contractions. Although the nature of these responses was not determined, the kinetics of these contractions and their sensitivity to capsaicin suggests the involvement of tachykinins released from capsaicin-sensitive neurones (Lundberg, Saria, Brodin, Rossell & Folkers, 1983). Indeed, histochemical studies have demonstrated that the rostral portion of the guinea-pig trachea receives its tachykinin-containing innervation from the superior laryngeal nerves (Baluk & Gabella, 1989). The infrequency with which these contractions occurred in preparations in which the oesophagus was left intact (and thus the vagal relaxant innervation intact) suggests that under normal circumstances these contractions elicited by vagus nerve stimulation may be functionally antagonized by the vagal relaxant innervation.

The role of the oesophagus in vagally mediated relaxations

Removing the oesophagus from our preparation selectively abolished vagally mediated relaxations of the guinea-pig trachea. Vagally mediated cholinergic contractions and adrenergic relaxations elicited by stimulation of the sympathetic trunks were unaffected. Although non-adrenergic relaxations could not be elicited by vagus nerve stimulation in preparations lacking an oesophagus, EFS (in the presence of propranolol) of these preparations produced non-adrenergic relaxations that were not different from those elicited in preparations having an oesophagus. The results support the hypothesis that the axons of the capsaicin-sensitive neurones, and the pre- and/or postganglionic axons of the parasympathetic neurones mediating non-adrenergic relaxations are in some way associated with the oesophagus prior to innervating the trachealis. The data also lead to the speculation that the trachea and oesophagus are functionally integrated.

An alternative explanation for the observation that oesophagus removal abolished vagally mediated, non-adrenergic relaxations of the trachealis is that the oesophagus

caused an *apparent* relaxation of the trachea by mechanically moving the trachea during vagus nerve stimulation. Still another explanation for these results is that upon vagus nerve stimulation the oesophagus released a substance that diffused to the trachea and relaxed the trachealis. In both of these cases, the non-adrenergic nerve terminals are envisaged to reside only in the oesophagus, with the trachealis relaxing to vagus nerve stimulation only as a consequence of the oesophageal innervation. There are several reasons why we consider these scenarios unlikely. First, intratracheal lidocaine or tetrodotoxin abolished tracheal dilatation elicited by vagus nerve stimulation while the oesophageal response was unaffected. This finding along with the fact that non-adrenergic relaxations are readily elicited by EFS of the trachea (without the oesophagus present) indicate that the relevant nerve endings are indeed in the trachea. Second, if the oesophagus were mechanically influencing the trachea or releasing some diffusable factor that relaxed the trachea, simple blunt dissection in the space between the oesophagus and trachea should not have abolished the relaxations elicited by vagus nerve stimulation. Third, no aspect of the oesophageal response to vagus nerve stimulation could be correlated kinetically with the relaxations of the trachealis. Finally, the functional response of the oesophagus to vagus nerve stimulation could be substantially inhibited, and in some experiments abolished, by atropine and succinylcholine while the vagally mediated relaxations of the trachea persisted.

The results indicate that the extrinsic pathways of the vagally derived relaxant innervation of the guinea-pig trachealis can be disrupted by removing the oesophagus. The experiments carried out, however, provide few insights into the precise anatomy of these pathways. Nevertheless, it is likely that the relaxant fibres bridge the oesophageal and tracheal tissues in the region of the trachealis that is ultimately innervated. This speculation is based on the finding that simple blunt dissection between the trachea and oesophagus adjacent to tracheal rings 3–9 abolished all vagally mediated relaxations of our preparations. Moreover, removing the oesophagus contiguous with the caudal half of the trachea had no effect on the tracheal relaxations. Indeed, it is worth noting that when viewed through a dissecting microscope, multiple neural projections can be seen spanning the gap between the oesophagus and trachea. Regardless of the precise anatomy of the non-adrenergic relaxant innervation, the finding that removing the oesophagus abolishes all vagally mediated relaxations may explain why these relaxations are seen in *in situ* preparations (Chesrown *et al.* 1980; Yip *et al.* 1981), but not in *in vitro* preparations (Blackman & McCaig, 1983; Widmarck & Waldeck, 1984; Pendry & MacClagen 1991). In the latter, the trachea is usually trimmed free of all extraneous tissue including the oesophagus.

The observation that parasympathetic contractions and relaxations of the guinea-pig trachea are differentially sensitive to oesophagus removal casts some doubt on the hypothesis that the contractile (acetylcholine) and the relaxant neurotransmitters of the parasympathetic innervation of the trachea function as co-transmitters. Rather, it would seem likely that the parasympathetic relaxant innervation of the guinea-pig trachealis is an autonomic system capable of functioning independently of the parasympathetic contractile innervation.

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