Innervation of tracheal parasympathetic ganglia by esophageal cholinergic neurons: evidence from anatomic and functional studies in guinea pigs

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Mazzone SB, McGovern AE. Innervation of tracheal parasympathetic ganglia by esophageal cholinergic neurons: evidence from anatomic and functional studies in guinea pigs. Am J Physiol Lung Cell Mol Physiol 298: L404–L416, 2010. First published January 8, 2010; doi:10.1152/ajplung.00166.2009.—In the present study, we describe a subset of nerve fibers, characterized by their immunoreactivity for the calcium-binding protein calretinin, that are densely and selectively associated with cholinergic postganglionic neurons in the guinea pig tracheal ganglia. Retrograde neuronal tracing with cholera toxin B, combined with immunohistochemical analyses, showed that these nerve fibers do not originate from sensory neurons in the nodose, jugular, or dorsal root ganglia or from motor neurons in the nucleus ambiguus, dorsal motor nucleus of the vagus nerve, spinal cord, stellate ganglia, or superior cervical ganglia. Calretinin-immunoreactive nerve fibers disappeared from tracheal segments after 48 h in organotypic culture, indicating that the fibers were of extrinsic origin. However, calretinin-positive nerve fibers persisted in tracheal ganglia when tracheae were cocultured with the adjacent esophagus intact. Immunohistochemical analysis of the esophagus revealed a population of cholinergic neurons in the esophageal myenteric plexus that coexpressed calretinin. In functional studies, electrical stimulation of the esophagus in vitro evoked measurable contractions of the trachea. These contractions were not altered by prior organotypic culture of the trachea and esophagus to remove the extrinsic innervation to the airways but were significantly (P < 0.05) inhibited by the ganglionic blocker hexamethonium or by physical disruption of the tissue connecting the trachea and esophagus. These data suggest that a subset of esophageal neurons, characterized by the expression of calretinin and acetylcholine, provide a previously unrecognized excitatory input to tracheal cholinergic ganglia in guinea pigs.

airway innervation; parasympathetic nervous system; gastroesophageal reflux disease

IT IS WELL ESTABLISHED that visceral parasympathetic postganglionic neurons are not simply a relay site for preganglionic activity. Rather, the intrinsic electrophysiological properties of specific parasympathetic postganglionic neurons allow for varying degrees of processing and filtering of preganglionic input (40, 42). In addition, synaptic efficacy is modulated by other neural (and hormonal) sources, including inputs arising from sensory, sympathetic, and other postganglionic neurons (5, 13, 39, 41, 43, 50). Two distinct subpopulations of parasympathetic postganglionic neurons innervate the guinea pig airways. Cholinergic (contractile) postganglionic neurons originate exclusively in the intrinsic airway ganglia, many of which are located on or near the adventitial surface of the trachea. By contrast, noncholinergic (relaxant) neurons originate in the adjacent (ventral) esophagus, where they are found intermingled with myenteric plexus neurons (20). Surprisingly, compared with many other visceral autonomic ganglia, little is known about the modulation of parasympathetic ganglionic transmission in the airways.

As predicted from numerous functional studies, tracheal parasympathetic ganglia are densely innervated by cholinergic nerve fibers that, presumably, originate from vagal preganglionic neurons in the brain stem or from intrinsic neurons from neighboring tracheal ganglia (although evidence for the latter is limited) (39, 53). Immunohistochemical studies have also shown that tracheal ganglia contain nerve fibers that express several neuropeptides, including substance P, calcitonin gene-related peptide, and galanin, commonly associated with vagal sensory nerves (13, 27, 33, 49), as well as fibers that express neuronal nitric oxide synthase (nNOS) and VIP (thought to be the esophageal-derived relaxant neurons) (19, 53). Consistent with this, functional studies have shown that substance P or the C-fiber stimulant capsaicin increases ganglionic transmission via neurokinin receptors located postsynaptically on intrinsic neurons (13), whereas NO and VIP reduce transmission through the ganglia (2). There is no evidence for tyrosine hydroxylase-positive nerve fibers (a marker for sympathetic postganglionic neurons) innervating the guinea pig tracheal ganglia (6, 33), although circulating catecholamines can undoubtedly modify ganglionic transmission (5, 50).

The calcium-binding proteins are a diverse family of proteins that are thought to be involved in buffering and sequestering of intracellular calcium. These proteins may also be useful markers for differentiating neuronal subtypes in the nervous system, inasmuch as different neurons generally display a distinct profile of calcium-binding protein expression. Accordingly, calcium-binding protein expression has been successfully used to distinguish common groups of neurons in the peripheral and central neuronal systems, including parasympathetic and sympathetic sensory and motor neurons that regulate visceral function (1, 7, 10, 21, 26, 28, 44, 48). In experiments attempting to further subclassify airway neuron subtypes in guinea pigs on the basis of calcium-binding protein expression, we noticed that the tracheal cholinergic ganglion cells were densely innervated by nerve fibers that express the calcium-binding protein calretinin. In the present study, we describe the origin of these fibers and provide anatomic and functional evidence for a previously unrecognized excitatory neural projection to the airway parasympathetic ganglia that originates in the nearby esophageal myenteric plexus.

MATERIALS AND METHODS

All experiments conducted were approved by accredited institutional Animal Ethics Committees. Experiments were performed on male albino Hartley guinea pigs (200–350 g body wt, n = 49; Institute of Medical and Veterinary Science, South Australia).

Preparation of tracheal and esophageal whole mounts. Whole-mount preparations of guinea pig tracheal (n = 8 animals) and
esophageal (n = 5 animals) segments were prepared as previously described (37, 38). Briefly, animals were deeply anesthetized with pentobarbital sodium (100 mg/kg ip) and transcardially perfused with 500 ml of 10 mM PBS. The entire trachea or esophagus was removed, cleaned of excess connective tissue, and opened longitudinally via a midline incision (along the ventral surface for the trachea and the dorsal surface for the esophagus). A cotton swab was used to gently rub the epithelium off the trachea. The esophageal mucosa and submucosa were separated from the underlying muscle layers by sharp dissection. Tissues (trachea, esophageal mucosa, and muscle layers) were pinned flat onto a piece of cork board, placed in fixative (4% paraformaldehyde) for 2–3 h at 4°C, and then transferred to blocking solution (10 mM PBS and 10% horse serum) for 1 h before immunohistochemical staining (see below). In some instances (for higher-resolution visualization of the myenteric ganglia), small segments of esophageal whole mounts were frozen in optimum cutting temperature (OCT) embedding medium and sectioned longitudinally (16 μm thick) using a cryostat. Sections were mounted on glass microscope slides (Super Frost Plus, Thermoscientific, Sydney, Australia) and processed for immunohistochemical markers as described below and listed in Table 1.

Retrograde tracing. Guinea pigs (n = 12) were anesthetized with ketamine (50 mg/kg ip) and xylazine (2 mg/kg ip). The extrathoracic trachea was exposed via a ventral incision in the animal’s neck, and the trachea was gently lifted away from the adjacent esophagus and surrounding tissue to provide access to the dorsal side of the trachea (i.e., the adventitial surface of the trachealis, where cholinergic ganglia are located). Using a 10-μl Hamilton glass microsyringe fitted with a 32-gauge needle and mounted on a micromanipulator, we slowly injected 2 μl of a 2% solution of cholera toxin B subunit (CTb) conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) into the tracheal adventitia at three to four rostrocaudal locations. Great care was taken not to allow the tracer to spill onto adjacent tissues or the nearby recurrent laryngeal nerves. Nevertheless, given the intimate relationship between the trachea and esophagus, some nonspecific transfer of tracer between the two tissues was unavoidable (evident in postexperiment examination of the tissues). In five additional experiments, 10 μl of CTb were injected via a ventral approach into the extrathoracic tracheal lumen (onto the mucosal surface). These latter experiments were performed to ensure that calretinin expression was assessed in all previously defined extrinsic nerve pathways. After injection, the wound was sutured. After 7 days of recovery, the animals were anesthetized with pentobarbital sodium (100 mg/kg ip) and transcardially perfused with 10 mM PBS followed by 4% paraformaldehyde in PBS. The medulla oblongata, entire spinal cord, and superior cervical, stellate, nodose, jugular, and/or dorsal root ganglia were removed and placed in 4% paraformaldehyde at 4°C for 2 h and then cryoprotected in 20% sucrose solution at 4°C overnight.

Immunohistochemical staining and microscopy. Immunohistochemical staining procedures were carried out as previously described (37, 38). For whole mounts, tissues were pinned flat to a Sylgard-filled tissue culture dish and incubated for 1 h in blocking solution (10% normal horse serum in 10 mM PBS) and then overnight (at 37°C) in 10 mM PBS-0.3% Triton X-100–2% horse serum containing the primary antiserum of interest (Table 1). After the whole mounts were washed thoroughly with 10 mM PBS (for ≥3 h), they were incubated for 1 h at room temperature in the appropriate Alexa Fluor-conjugated secondary antiserum (Table 1). For immunohistochemical processing of tissue sections, nodose and jugular ganglia and stellate, dorsal root, and superior cervical ganglia were rapidly frozen in OCT embedding medium, and 16-μm cryostat-cut sections were mounted directly onto glass slides. Brain stems and segments from the cervical, thoracic, and lumbosacral spinal cord were frozen in OCT using CO2 and cut (at 40 μm) using a Leica freezing microtome, and the free-floating sections were processed. The same immunohistochemical methods were used for slide-mounted and free-floating sections. All sections were incubated for 1 h in blocking solution (10% horse serum) and then overnight (at room temperature) in PBS-0.3% Triton X-100–2% horse serum and the primary antiserum of interest (Table 1). Sections were washed several times with PBS and then incubated with the appropriate Alexa Fluor-conjugated secondary antiserum (Table 1). Free-floating sections were mounted on glass slides, and coverslips were applied to all sections with buffered glycerol.

Labeling of whole mounts and slide-mounted sections was visualized using an Olympus BX51 fluorescence microscope equipped with appropriate filters and an Optronics digital camera. Negative control experiments, in which the primary antisera were excluded, were carried out where necessary. Cell counts in a given field of view were performed at ×200 magnification using 3–10 representative replicate sections from ≥4 animals. Values are means ± SE.

Organotypic tissue cultures. Organotypic cultures of guinea pig trachea were obtained as previously described (15). Guinea pigs (n = 14) were deeply anesthetized with pentobarbital sodium (100 mg/kg ip) and transcardially perfused with 10 mM PBS. The trachea and adjacent esophagus were removed en bloc and quickly submerged in cold, sterile MEM with Earle’s salts and l-glutamine (Sigma, Australia). Care was taken to remove all excess connective tissue and all extrinsic nerves. Tracheae, in the absence (n = 5) or presence (n = 9) of the adjoining esophagus, were cultured for 48 h at 37°C in sterile, carbogen-gassed MEM containing 10 μM indomethacin, 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma, Australia). The culture medium was changed every 12 h, and after 48 h the tracheae were processed for whole-mount immunohistochemical staining as described above.

Table 1. Primary and secondary antibodies

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<td>Goat</td>
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<td>Mouse</td>
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<td>MAb 5254 (Chemicon, Vic, Australia)</td>
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<tr>
<td>nNOS</td>
<td>Sheep</td>
<td>1:4,000</td>
<td>Gift of Dr. Colin Anderson (University of Melbourne, Australia)</td>
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<tr>
<td>SP</td>
<td>Rat</td>
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<td>MAb 356 (Chemicon, Vic, Australia)</td>
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**Secondary [IgG (H + L), 2 mg/ml]**

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ChAT, Choline acetyltransferase; nNOS, neuronal nitric oxide synthase; SP, substance P.
scribed above (n = 5 cultures without esophagus and n = 5 cultures with esophagus attached) or used for functional studies as described below (n = 4 cultures with esophagus attached).

In vitro functional studies. Cholinergic-mediated contractions of the guinea pig trachea were measured using an in vitro tracheal tube preparation. Experiments were carried out using freshly excised tissues (n = 14) or tissues that had been maintained in organotypic culture for 2 days (as described above; n = 4). For freshly excised tissues, guinea pigs were deeply anesthetized with pentobarbital sodium (100 mg/kg ip) and exsanguinated. The trachea and adjacent esophagus were removed en bloc and pinned to the base of a Sylgard-lined water-jacketed dissection dish that was continuously overfilled with warmed (37°C), oxygenated Krebs bicarbonate buffer (in mM: 118 NaCl, 5.4 KCl, 1 NaHPO₄, 1.2 MgSO₄, 1.9 CaCl₂, 25
NaHCO₃, and 11.1 dextrose); 3 μM indomethacin (Sigma, Australia) was added to the buffer to prevent any potential local effects of prostaglandins on the tissues under study. The tissues were then cleaned of any excess connective tissue, and all extrinsic nerves were removed. Care was taken not to disrupt the tissue between the trachea and esophagus. Cannulas (15-gauge Luer stubs bent at right angles) were sutured into the caudal- and distalmost ends of the tracheal tube: the distal cannula was connected to a syringe containing Krebs buffer for filling of the tracheal lumen, and the caudal end was attached to a pressure transducer for measurement of static intraluminal tracheal pressure. Three-way taps were incorporated into the circuit to enable filling of the trachea and removal of any air bubbles. Output from the pressure transducer was amplified and filtered (Neurosys Systems, Digitimer, Hertfordshire, UK), digitized (Micro1401 A-D converter, CED, Cambridge, UK), and recorded using Spike II software (CED). In preliminary experiments, we determined the optimum baseline intraluminal pressure (5–10 mmHg) for measurement of contractions of the trachea after vagus nerve or electric field stimulations (data not shown), and this baseline pressure was used for all subsequent experiments. At 30 min before each experiment was started, the following drugs (in addition to indomethacin) were added to the perfusion buffer: 2 μM propranolol (Sigma, Australia) to prevent any sympathetic-mediated relaxations (35), 0.1 μM CP-99994 and SR-48968, and 0.3 μM SB-222200 (generous gifts from Glaxo-SmithKline, King of Prussia, PA) to prevent any confounding effects of tachykinins acting at neurokinin (NK₁, NK₂, or NK₃) receptors (35), 1 μM HH-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (Sigma, Australia), a soluble guanylate cyclase inhibitor, to limit the relaxant effects of neurally released NO (29), and 1 μM Rp-cAMP, the cAMP inhibitor, to limit the relaxant effects of neurally released VIP (dose determined in preliminary pharmacological experiments). Cultured tissues were prepared for functional studies using the same protocol.

In the first series of experiments using freshly excised tissues (n = 5), a custom-built bipolar silver wire (125 μm diameter; SDR Clinical Technology, Sydney, Australia) stimulating electrode was placed into the lumen of the esophagus, such that the rostral two-thirds of the lumen was in contact with the electrode. A stimulator (model s48, Grass Instruments) was used to deliver increasing voltages (1–50 V) to the esophagus at a constant stimulating frequency (32 Hz), pulse duration (1 ms), and train duration (10 s). Then, at a voltage that evoked reproducible responses at ~30–40% of the maximum, we compared the average magnitude of contractions before and after bath application of vehicle (distilled water), the ganglionic blocker hexamethonium (100 and 300 μM) (14), and the muscarinic receptor antagonist atropine (1 μM) (35). In a separate series of experiments employing freshly excised (n = 5) or cultured (n = 4) tissues, we compared esophageal stimulation-evoked contractions before and after physical disruption of the tissue interconnecting the trachea and esophagus. In these experiments, great care was taken to maintain the relative position of the trachea and esophagus after dissection. Thus tissues remained in physical contact, with only the bridging connective tissue (and the putative axonal projections) disrupted. In control experiments (n = 4), a custom-built stainless steel wire (360 μm diameter; WPI, Sarasota, FL) field stimulating electrode straddled either side of the tracheal tubes. Voltage-response curves were assessed, and the effects of vehicle, hexamethonium, and atropine were compared at approximately half-maximum voltages. At the end of every experiment, the maximum attainable contraction of the trachea was determined by addition of 300 mM BaCl₂ to the buffer, and the magnitude of the responses was expressed as a percentage of this maximum contraction. Values are means ± SE, and data were compared statistically using one-way ANOVA followed by Dunnett’s multiple-comparisons method.

RESULTS

Calretinin expression in guinea pig trachea. Immunohistochemical staining for choline acetyltransferase (ChAT) in whole-mount preparations of guinea pig trachea revealed an organized network of nerve fibers interconnecting groups of intrinsic tracheal ganglion cells (Fig. 1A). Cholinergic ganglia were found exclusively on the adventitial surface of the trachea and consisted of 2–25 cells. The majority (>80%) of ganglion cells were densely surrounded by nerve fibers immunoreactive for calretinin (Fig. 1, B–E). Although on close inspection it appeared that many calretinin-positive nerve fibers also stained positive for ChAT [although this was difficult to ascertain with confidence using whole-mount preparations and regular fluorescence microscopy (data not shown)], there was clearly no expression of calretinin in cholinergic ganglion cell soma. Furthermore, there were no calretinin-positive nerve fibers in or near the mucosal surface of the trachea, nor was there any obvious staining of fibers in the tracheal smooth muscle [although ChAT-positive fibers, presumably from cholinergic postgangionic neurons, were readily visible in the muscle (Fig. 1F)]. Calretinin-positive nerve fibers in the tracheal ganglia were not immunoreactive for common markers of other subtypes of airway nerves, including substance P, neurofilament, the α₁-subunit of Na⁺-K⁺-ATPase, VIP (data not shown), or NOS (see below).

Retrograde tracing of sensory and motor neurons innervating the trachea. Retrograde neuronal tracing from the tracheal adventitia identified populations of neurons in the nodose, jugular, and dorsal root ganglia (sensory neurons) and the stellate and superior cervical ganglia (sympathetic postganglionic neurons) and the compact formation of the nucleus ambiguus (nAmb) and the caudal (obex level) dorsal motor nucleus of the vagus nerve (dmnX) in the brain stem (vagal preganglionic neurons; Fig. 2). Retrograde tracing from the tracheal mucosa only labeled neurons in the nodose and jugular ganglia. Calretinin was expressed in a small number of cells and nerve fibers in each of these locations (Table 2); however, there was no expression of calretinin in any of the neuronal populations traced from the trachea (Fig. 2).

Although we did not perform detailed characterization and comparison of vagal ganglia neurons labeled via adventitial vs. tracheal lumen tracer injections, we did not observe any obvious differences in the types of neurons traced. For example, nodose neurons traced from the adventitia or lumen were of comparable sizes and expressed neurofilament. Similarly, jugular ganglia neurons were large and expressed neurofilament, or they were small and substance P-positive (data not shown). This is consistent with the well-described sensory innervation to the guinea pig trachea (12, 33, 36, 46). In the nodose and jugular ganglia, calretinin-positive cells represented <2.5% of the total cell population (Table 2). These cells were typically medium- to large-diameter myelinated neurons (i.e., they expressed neurofilament but not substance P; Fig. 2, A–F, Table 2). However, only a small percentage of neurofilament-positive (myelinated) neurons in the vagal sensory ganglia expressed calretinin (Fig. 2, B and E, Table 2). In cross sections of the recurrent laryngeal nerve, large-diameter calretinin-positive axons were visible, presumably the axons of calretinin-positive neurons in the vagal ganglia (not shown). In the brain stem, most neurons in the compact nAmb and caudal dmnX (includ-
ing those traced from the tracheal adventitia) stained positive for ChAT (Table 3). No brain stem neurons were retrogradely labeled after luminal tracer injections. In total, among >1,200 nAmb cells, only 3 calretinin-positive cells were observed, and 2 of these did not colocalize ChAT (Fig. 2J, Table 3). By contrast, a population of calretinin-positive cells was consistently observed in the dmnX, especially in its more rostral regions (Fig. 2K). Although many of these neurons were typically small diameter and did not coexpress ChAT, a distinct ChAT-positive group of calretinin-expressing cells was
also present (Fig. 2K, Table 3). The calretinin-positive cells in the dorsal root, superior cervical, and stellate ganglia were not further characterized.

In addition to the expression of calretinin in cell soma, calretinin-positive nerve terminals were also present in the nodose ganglia (Fig. 2B), dmnnX, nAmb, and stellate and superior cervical ganglia (Fig. 2, G–I). These terminals typically formed dense basket-like structures around cell soma in each of these locations. In the stellate and superior cervical ganglia, calretinin-positive pericellular baskets were frequently observed around CTb-positive neurons retrogradely traced from the trachea (Fig. 2J), suggesting that some sympathetic postganglionic neurons that project to the trachea are also innervated by calretinin-positive nerve fibers [possibly the terminals of sympathetic preganglionic neurons originating from the spinal cord, inasmuch as they have been shown to express calretinin in previous studies (21)]. We did not see any neurons in the intermediolateral cell column after retrograde tracer injection into the tracheal adventitia (data not shown), suggesting that sympathetic preganglionic neurons do not project to tracheal ganglia (and, therefore, are not likely the source of calretinin in the trachea).

**Esophageal origin of calretinin-positive nerve fibers innervating tracheal ganglia.** ChAT immunoreactivity in intrinsic ganglia persisted when tracheal whole mounts were cultured in organotypic media for 48 h with the esophagus removed (Fig. 3A). Strips of cultured trachea retained cholinergic contractions to field stimulation in vitro (peak contractions at optimum stimulating frequencies occurred at 40–50 V and were 69–88% of the maximum attainable contraction in control and cultured tracheae), confirming the viability of the tissues after organotypic culture (in particular, the airway cholinergic postganglionic neurons). However, after the tissues were cultured, there was a total absence of calretinin-positive nerve fibers innervating the ganglia (Fig. 3A). By contrast, calretinin-positive nerve fibers were readily observed innervating cholinergic ganglia cells in tracheae that were cultured for 48 h with the adjacent esophagus intact (Fig. 3B).

Immunohistochemical staining of esophageal whole mounts and thin sections revealed a subset of myenteric plexus neurons that were intensely fluorescent for calretinin immunoreactivity (Fig. 3, C–E). Calretinin-expressing neurons displayed dense dendritic branching (Fig. 3D) or long filamentous processes that could be followed over many millimeters of tissue (Fig. 3E). Calretinin-expressing neurons tended to be located on the perimeter of myenteric ganglia or intermingled with the bundles of nerve fibers that interconnected adjacent ganglia (e.g., whole-mount preparations in Figs. 3C and 4A). It was also apparent that, similar to the tracheal ganglia, myenteric ganglia neurons were densely innervated by calretinin-expressing nerve fibers (Figs. 3C and 4A).

Calretinin-positive neurons in the myenteric plexus never coexpressed nNOS (0 of 181 calretinin-positive neurons, n = 4 experiments). Furthermore, nNOS-positive neurons never expressed calretinin (0 of 483 nNOS-positive neurons, n = 4 experiments; Fig. 4, A–D). Interestingly, nNOS-positive neurons in the esophagus were also typically in close association with calretinin-positive nerve fibers (Fig. 4D). In the tracheal ganglia, nNOS-positive nerve fibers (unlike calretinin-positive fibers) were only sparsely distributed among ganglia neurons, and those fibers never expressed calretinin (Fig. 4C). In esophageal whole mounts, it proved difficult to successfully visualize ChAT immunoreactivity in myenteric neurons [similar problems have also been reported by others (16)]. However, subsets of ChAT-positive neurons were observed in thin longitudinal and cross sections of the esophagus. Although not quantitatively assessed in the present study, most (but not all) neurons that expressed calretinin also displayed detectable levels of ChAT immunoreactivity (Fig. 4, D and E). In one additional experiment (data not shown), calretinin was also seen in neurons that were immunoreactive for the vesicular acetylcholine transporter (an alternative marker for cholinergic neurons).

**Functional studies.** Electrical stimulation of the esophagus evoked voltage-dependent contractions of the trachea in vitro (Figs. 5A and 6). Esophageal-evoked contractions of the trachea.
chea were readily observed in freshly excised and cultured tissues (Fig. 7). The threshold and half-maximum voltages averaged 39.1 ± 5.1 and 54.3 ± 2.5 V, respectively, and the maximum esophageal-evoked contractions approximated the maximum attainable contraction produced by addition of BaCl₂ to the bath (n = 10 preparations). The average magnitude of contractions evoked using submaximum stimulation voltages was unaffected by the addition of vehicle (100 μl of distilled water) to the bath but was substantially reduced in four of five preparations by the addition of 100 μM hexamethonium (average inhibition for the 5 preparations was 61.5 ± 14.6%; Figs. 5A and 6, A and B). No further inhibition was achieved by increasing the bath concentration of hexamethonium to 300 μM. The remaining hexamethonium-resistant component of the contractions was, however, abolished in the presence of 1 μM atropine (Fig. 5A).

In freshly excised tissues, electric field stimulation of the trachea also evoked voltage-dependent contractions (Figs. 5B and 7, inset). The threshold and half-maximum voltages averaged 22.5 ± 2.5 and 38.8 ± 3.8 V, respectively, and the maximum field stimulation-evoked contractions approximated the maximum attainable contraction produced by addition of BaCl₂ to the bath (n = 5 preparations). Organotypic culture did not alter field stimulation-evoked contractions (Fig. 7, inset), confirming the appropriateness of the culture conditions. At submaximum voltages, field stimulation-evoked contractions were unaffected by bath application of vehicle.

Table 3. Calretinin expression in parasympathetic motor neurons in the nAmb and dmnX

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<th>dmnX</th>
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<td>0.2 ± 0.1 (3/1,271)</td>
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<td>CTb-positive</td>
<td>0 ± 0 (0/284)</td>
<td>0 ± 0 (0/41)</td>
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<td>99.4 ± 0.3 (299/301)</td>
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<td>Calretinin-positive, ChAT-positive</td>
<td>0.1 ± 0.1 (1/1,269)</td>
<td>4.3 ± 0.5 (72/1,583)</td>
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Values are means ± SE (n = 4 animals) expressed as number of positive neurons relative to total number of cells counted (shown in parentheses). nAmb, nucleus ambiguous; dmnX, dorsal motor nucleus of the vagus nerve.

Fig. 3. Esophageal origin of calretinin-immunoreactive nerve fibers in tracheal ganglia. A and B: representative calretinin (A, green) and ChAT (A', red) immunoreactivity in tracheal whole-mount preparations 48 h after organotypic cultures, without (A) or with (B) adjacent esophagus intact. Calretinin immunoreactivity only persisted when the trachea was cultured with the esophagus. In esophageal whole-mount preparations (C) and thin sections (D and E), neurons (arrows) intensely immunoreactive for calretinin were clearly identified in or near the myenteric plexus. Arrowheads in E highlight a long filamentous process extending from the calretinin-positive soma. Photos are representative of 5 similar experiments. Scale bars, 50 μm.
Fig. 4. Immunohistochemical characterization of esophageal calretinin-expressing neurons. In esophageal whole mounts (A–C) and thin sections (D), it was clear that calretinin-immunoreactive neurons (green, arrowheads) were never immunoreactive for neuronal nitric oxide synthase (nNOS, red), although nNOS-positive myenteric plexus neurons were closely associated with calretinin-immunoreactive nerve fibers (arrows). Calretinin and nNOS were also not colocalized in nerve fibers in tracheal whole mounts [E, arrowheads show calretinin nerve fibers around tracheal ganglia cells (1–3), while arrows show nNOS-positive nerve fibers coursing through the ganglia]. Calretinin-immunoreactive neurons in the myenteric plexus were immunoreactive for ChAT (F and G). Scale bars, 50 μm.
or 100–300 μM hexamethonium (Figs. 5B and 6, C and D). Contractions were, however, abolished in the presence of 1 μM atropine (Fig. 5B).

In other experiments, electrical stimulation of the esophagus at submaximum voltages (35–45 V) evoked contractions that were significantly (P < 0.05) reduced in magnitude after sharp dissection of the tissue interconnecting the trachea and esophagus. Thus, whereas reproducible contractions were evident in two successive bouts of electrical stimuli without tissue disruption (30.9 ± 7.7 and 36.6 ± 8.0% maximum contraction; Fig. 7), the average magnitude of tracheal contractions following dissection of the esophagus and trachea was significantly less (5.4 ± 3.3% maximum contraction; Fig. 7). Esophageal-evoked contractions in cultured tissues were also inhibited by dissection of the tissue interconnecting the trachea and esophagus (Fig. 7). Furthermore, in all tissues, after tissue dissection, hexamethonium had no further inhibitory effect on esophageal-evoked tracheal contractions or on tracheal contractions evoked by electric field stimulation (not shown).

**DISCUSSION**

The results of the present study show that tracheal cholinergic ganglia in guinea pigs are densely innervated by a group of nerve fibers that express the calcium-binding protein calretinin. Retrograde tracing and immunohistochemical analyses revealed that these nerve fibers do not originate from sensory neurons in the nodose, jugular, or dorsal root ganglia, sympathetic preganglionic neurons in the spinal cord, sympathetic postganglionic neurons in the stellate or superior cervical ganglia, or vagal preganglionic neurons in the dmnX or nAmb. Rather, evidence obtained using organotypic culture suggests that calretinin-positive nerve fibers originate from a subset of neurons in the adjacent esophagus. Immunohistochemical analysis of the esophagus revealed a population of calretinin-positive neurons in the myenteric plexus. Unlike other esophageal neurons that have been shown to project to the airways (19), these neurons did not express nNOS but, rather, expressed the cholinergic marker ChAT. Consistent with this, electrical stimulation of the esophagus in vitro evoked contractions of the trachea that were inhibited by administration of the ganglionic blocker hexamethonium or by dissection of the tissue interconnecting the trachea and esophagus. These data suggest that a previously unrecognized population of cholinergic esophageal neurons provide excitatory innervation to intrinsic tracheal cholinergic ganglia in guinea pigs.

Calretinin-positive fibers innervating tracheal cholinergic ganglia. The neuronal expression of calcium-binding proteins in the airways has been poorly assessed. In rats, calretinin has been shown to be expressed by a subset of structurally complex vagally derived sensory nerve terminals on the mucosal surface.
of the trachea and bronchi (52). Although the functional identity of these terminals was not characterized, they were thought to represent slowly adapting mechanoreceptors (stretch receptors) in these species (52). Similarly, other types of mechanoreceptors in rats may also express calretinin (3, 17, 32). Of particular relevance, calretinin is expressed by vagally derived slowly adapting mechanosensitive nerve fibers innervating esophageal ganglia (so-called intraganglionic laminar endings) (18, 32, 51). However, we failed to find any evidence in the present study that the calretinin-positive nerve fibers in the guinea pig tracheal ganglia are of sensory origin. In guinea pigs, most sensory neurons projecting to the trachea are derived from the nodose and jugular ganglia, and only very few originate in the dorsal root ganglia (33, 38; present study). First and foremost, we observed very few calretinin-positive neurons in any of the sensory ganglia. Second, we never saw calretinin expression in any sensory neurons that were retrogradely traced from the tracheal adventitia or the tracheal lumen, despite our tracing experiments identifying neurons from all the known subclasses of tracheal afferents. This is consistent with our previous studies showing an absence of calretinin in nodose neurons retrogradely traced from the guinea pig tracheal lumen using Fluoro-Gold (38). Finally, we never saw calretinin-positive nerve terminals on the mucosal surface of tracheal whole mounts (where many airway sensory neurons terminate), and calretinin-positive fibers in the airway ganglia did not coexpress markers that identify afferent neurons in the guinea pig airways (33, 38, 46). Interestingly, the guinea pig trachea is devoid of slowly adapting stretch receptors (12), perhaps providing one explanation for the lack of calretinin-positive sensory nerve terminals in this tissue. Whether any of the small population of unidentified myelinated calretinin-positive neurons in the vagal ganglia represents the soma of intrapulmonary stretch receptors is unknown. Alternatively, rats and guinea pigs may simply differ with respect to sensory neuron expression of calretinin.

Calretinin-positive nerve fibers in the tracheal ganglia were also not derived from vagal preganglionic, parasympathetic postganglionic, or sympathetic postganglionic neurons. As reported by others, retrograde tracing from the tracheal adventitia (but not tracheal lumen) identified cholinergic neurons in two brain stem nuclei, the dmX and the nAmb, as well as sympathetic neurons in the stellate and superior cervical ganglia (4, 22, 33). Populations of calretinin-positive neurons and nerve fibers were identified at each of these locations; however, calretinin was never expressed in neurons that were retrogradely traced from the airways. In the brain stem, very few calretinin-positive neurons were present in the nAmb, whereas those in the dmX were often relatively small in diameter, perhaps representing interneurons at this site. In the sympathetic ganglia, it was not uncommon to observe neurons that were retrogradely traced from the trachea receiving dense pericellular basket-like innervation from calretinin-positive nerve terminals. A similar observation has been reported for neuropeptide Y-negative superior cervical ganglia neurons that innervate the rat submandibular salivary gland and heart, suggesting that a subset of sympathetic preganglionic neurons may selectively express calretinin (21, 47). In our study,
muscle) and immunohistochemistry experiments showed that organs (19). Retrograde tracing (from the tracheal smooth muscle via the tissue that interconnects these fibers persisted in the tracheal ganglia. In guinea pigs, a cultured with the esophagus intact, calretinin-positive nerve fibers terminate), it was impossible to selectively trace this intrinsic cholinergic neurons themselves) disappeared. This possibility that calretinin-positive sympathetic preganglionic neurons provide direct input to tracheal parasympathetic ganglia neurons in guinea pigs. Cholinergic neurons in the tracheal ganglia were also devoid of calretinin immunoreactivity, indicating that the calretinin-positive terminals do not arise from neighboring tracheal ganglia.

Esophageal origin of calretinin-positive fibers innervating tracheal cholinergic ganglia. Unfortunately, given the intimate anatomic relationship and shared blood supply of the esophagus and tracheal adventitia (where calretinin-expressing nerve fibers terminate), it was impossible to selectively trace this nerve pathway using standard neuroanatomic tracing techniques. For this reason, we performed nerve degeneration experiments by assessing the effect of organotypic culture on calretinin expression in the trachea. When the trachea was maintained in culture with the esophagus removed, calretinin-positive nerve fibers innervating the tracheal ganglia (but not the intrinsic cholinergic neurons themselves) disappeared. This confirms that calretinin-expressing fibers originate from an extrinsic neuronal source. Interestingly, when the trachea was cultured with the esophagus intact, calretinin-positive nerve fibers persisted in the tracheal ganglia. In guinea pigs, a population of postganglionic neurons resides in the neighboring esophagus and projects axons to the tracheal ganglia and tracheal smooth muscle via the tissue that interconnects these organs (19). Retrograde tracing (from the tracheal smooth muscle) and immunohistochemistry experiments showed that up to 58% of these esophageal-derived postganglionic neurons express nOS and VIP (19), suggesting that another population of esophageal neurons (not immunoreactive for nOS or VIP) likely exists. Consistent with this, calretinin-positive neurons were identified in the esophageal myenteric plexus, and neither these neurons nor the calretinin-expressing nerve fibers in the trachea expressed nOS. The intrinsic neurons of the esophageal myenteric plexus have not been characterized as carefully as have myenteric neurons in other regions of the gastrointestinal tract. However, evidence from intestinal ganglia suggests that two distinct types of neurons express calretinin in the myenteric plexus: ascending interneurons and motor neurons innervating the longitudinal muscle (9, 10, 45, 48). Both neuronal types coexpress the cholinergic marker ChAT but are differentiated by their projection patterns, physiology, and the expression of other markers such as neurofilament triplet protein (8). Interestingly, the projections of ascending interneurons can be traced many millimeters from their soma, and these neurons have been shown to evoke responses primarily via nicotinic cholinergic neurotransmission (9). We observed that the majority of calretinin-positive neurons in the myenteric plexus were also cholinergic. Indeed, the absence of nOS and the presence of ChAT in esophageal calretinin neurons support our assertions that these neurons represent a novel projection from the esophagus to the airways. Nevertheless, whether these neurons express other markers common to airway relaxant neurons (primarily VIP) and, hence, simply represent a subpopulation of relaxant neurons is unclear. Previous studies and the pattern of immunostaining in the tracheal ganglia, however, do not support this possibility. For example, VIP is not coexpressed in any known population of calretinin-positive neurons in the guinea pig gastrointestinal tract (9, 10). Furthermore, calretinin-immunoreactive fibers densely innervate airway ganglia (present study), whereas VIP-containing fibers are sparse (similar to the nOS labeling shown in Fig. 4C) (2). A careful analysis of the neurochemistry of the esophageal calretinin-expressing neurons that innervate the trachea will resolve this issue.

Our functional studies also support the existence of esophageal-derived cholinergic neurons innervating the tracheal cholinergic ganglia. Electrical stimulation of the esophagus in vitro evoked contractions of the trachea that were sensitive to ganglionic blockade with hexamethonium, the potency of which was identical to that reportedly needed to block vagus nerve stimulation-evoked contractions of guinea pig airways (14). This suggests that neurons arising from the esophagus provide cholinergic excitatory input to tracheal ganglia. Consistent with this, acute disruption of the tissue that normally connects the trachea and esophagus also significantly reduced esophageal-evoked contractions. A similar approach has been used previously to confirm the presence of esophageal nonadrenergic relaxant neurons innervating the trachea in guinea pigs (19).

The close proximity of the esophagus and trachea, of course, raises some concern that tracheal contractions in our in vitro preparation are secondary to the electrical current spreading directly to tracheal ganglia. However, ganglionic transmission is redundant during electric field stimulation of the trachea (hence, the insensitivity of contractions to hexamethonium in the present study). In addition, disruption of the interconnecting tissue (but repositioning of the trachea and esophagus in direct apposition) effectively reduced the magnitude of the responses. Nevertheless, we did routinely see a component of...
the tracheal contraction following esophageal stimulation that was not sensitive to hexamethonium but was abolished by atropine, suggesting that some current spread to the tracheal ganglia was unavoidable.

The functional studies conducted in freshly isolated tissues do not differentiate between responses mediated by stimulating intrinsic neurons and those mediated by stimulating fibers of an extrinsic origin that are in close proximity to the trachea and/or esophagus. For example, it may be possible that the functional data could be explained if the same preganglionic neurons innervate the esophageal and tracheal ganglia (i.e., via branching axons). Thus excitation of preganglionic terminals in the esophagus would lead to axon reflex-like preganglionic-mediated stimulation of tracheal ganglia. However, we previously employed retrograde tracing experiments to show that distinct neurons in the dmnX and nAmb project to the airways and esophagus (36). Furthermore, identical functional results were obtained using tracheae that had been maintained in organotypic culture with the esophagus intact. Inasmuch as organotypic culture is presumed to remove the extrinsic innervation to the airways without effecting intrinsic postganglionic responses (15; present study), these data would help confirm that the contractions evoked by esophageal stimulation likely involve a local circuitry between the two tissues. The lack of calretinin expression in vagal preganglionic neurons and the effects of organotypic culture on calretinin expression observed in the present study also support this hypothesis.

Functional significance. Esophageal neurons that innervate cholinergic neurons in the guinea pig tracheal ganglia may represent a mechanism regulating neurotransmission and postganglionic activity at this site. Previous studies have shown that, in addition to directly relaxing airway smooth muscle, esophageal-derived NOS/VIP relaxant postganglionic neurons provide inhibitory regulatory input to cholinergic postganglionic neurons (2, 14). However, unlike the NOS/VIP relaxant neurons that only sparsely innervate cholinergic ganglia in the trachea (2; present study), calretinin-positive cholinergic esophageal neurons densely innervate cells in the tracheal ganglia. It is also of interest that we did not observe any apparent innervation of the tracheal smooth muscle by calretinin-positive nerve fibers (although we did see NOS- and ChAT-positive fibers in the muscle layer). This may suggest that, unlike the NOS/VIP neurons and airway postganglionic cholinergic neurons that regulate ganglionic transmission and smooth muscle tone, the calretinin-positive neurons may represent a selective pathway controlling airway ganglionic activity only.

Given that the tracheal ganglia neurons contribute innervation to all levels of the airways, input from the esophageal pathway would conceivably have widespread effects on airway physiology (perhaps including airway and vascular smooth muscle tone and glandular activity). However, the circumstances under which esophageal cholinergic neurons are involved in regulating airway ganglionic activity are unclear. We do not know if esophageal neurons provide ongoing input to cholinergic neurons in the airways or whether they can be recruited in a local reflex manner to modify ganglionic output. However, the latter possibility may have important implications. Clinical studies in humans suggest that up to 80% of patients with asthma display concomitant signs of gastroesophageal reflux disease (23, 30, 34). In many asthmatic patients, at least some improvement of their asthmatic symptoms is achievable with effective pharmacological or surgical treatment of their reflux (24, 31). This has led to much debate about the possible interactions between the esophagus and the airways. Aspiration of gastric contents, infiltration of inflammatory cells via the vasculature that is shared by the esophagus and airways, acid-evoked sensory nerve axon reflexes, and classic sensory nerve-evoked brain stem reflexes have been implicated (for review see Refs. 11 and 33). Our data may provide an alternative mechanism whereby local reflexes initiated from the esophagus could conceivably amplify ongoing ganglionic activity in the airways via cholinergic mechanisms and lead to the onset of respiratory symptoms following reflux events. The documented effectiveness of anticholinergic drugs to reverse bronchial obstruction following esophageal acidification in asthmatic patients supports this suggestion (25). Nevertheless, it must be acknowledged that esophageal projections to the airways may be a peculiarity of the guinea pig, inasmuch as there is a paucity of evidence of comparable circuitry in humans.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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