Parasympathetic innervation of canine tracheal smooth muscle

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Valic, Zoran, Edward H. Vidruk, Stephen B. Ruble, John B. Buckwalter, and Philip S. Clifford. Parasympathetic innervation of canine tracheal smooth muscle. J Appl Physiol 90: 23–28, 2001.—To investigate whether efferent parasympathetic fibers to the tracheal smooth muscle course through the pararecurrent nerve rather than the recurrent or the superior laryngeal nerve, we stimulated all three nerves in anesthetized dogs. We also recorded the pararecurrent nerve activity response to bronchoconstrictor stimuli and compared it with pressure changes inside a saline-filled cuff of an endotracheal tube. Electrical stimulation (30 s, 100 Hz, 0.1 ms, 10 mA) increased tracheal cuff pressure by 21.0 ± 3.2 and 1.3 ± 0.7 cmH2O for the pararecurrent and the recurrent laryngeal nerve, respectively. Stimulation of the superior laryngeal nerve increased tracheal cuff pressure before, but not after, sectioning of the ramus anastomoticus, which connects it to the pararecurrent nerve. Intravenous administration of sodium cyanide increased pararecurrent nerve activity by 208 ± 51% and tracheal cuff pressure by 14.4 ± 3.5 cmH2O. Elevation of end-tidal P CO2 to 50 Torr increased pararecurrent nerve activity by 49 ± 19% and tracheal cuff pressure by 8.4 ± 3.6 cmH2O. Further elevation to 60 Torr increased pararecurrent nerve activity by 101 ± 33% and tracheal cuff pressure by 11.3 ± 2.9 cmH2O. These results lead us to the conclusion that parasympathetic efferent fibers reach the smooth muscle of the canine trachea via the pararecurrent nerve.

pararecurrent nerve; recurrent laryngeal nerve; tracheal smooth muscle tone; acetylcholine; parasympathetic preganglionic fibers

Parasympathetic innervation of the respiratory tract is potentially involved in pathogenesis of asthma because of bronchoconstrictor action of acetylcholine released from the nerve endings, which innervate smooth muscle along the tracheobronchial tree. There are substantial anatomic differences in parasympathetic innervation among different species. In humans, the trachea is innervated via the recurrent nerve, which carries parasympathetic fibers inside the same sheath as motor and sensory fibers innervating the larynx. Based on anatomic observations, Lemere (5, 6) described innervation of the canine trachea by a separate nerve called the pararecurrent nerve. Recently, Vidruk (10) described afferent fibers from tracheal stretch receptors coursing through the pararecurrent nerve. Although efferent fibers in this nerve were not investigated, he suggested the possibility that both sensory and motor fibers might travel within the pararecurrent nerve. Brown et al. (1) concluded that parasympathetic fibers to the canine trachea arise from the superior laryngeal, recurrent laryngeal, and pararecurrent nerves. However, they did not directly stimulate the pararecurrent nerve because they were unable to distinguish it as a separate structure. They also failed to take into consideration the anatomic connections between the superior laryngeal nerve and the pararecurrent nerve. We hypothesized that the majority of parasympathetic efferent fibers to the canine trachea travel through the pararecurrent nerve rather than recurrent laryngeal nerve or superior laryngeal nerve. To test this hypothesis, we used three approaches. We compared the tracheal constriction elicited by direct stimulation of the three different nerves and studied the level of constriction to intravenous administration of sodium cyanide before and after sectioning the nerves. We also recorded the changes in efferent nerve activity from the pararecurrent nerve to known bronchoconstrictor stimuli.

METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Animals. Nineteen mongrel dogs (12–20 kg) were used in this study. Anesthesia was induced with bolus intravenous infusion of 100 mg/kg α-chloralose and 500 mg/kg urethane and was maintained with continuous intravenous infusion of 20 mg·kg⁻¹·h⁻¹ α-chloralose and 100 mg·kg⁻¹·h⁻¹ urethane. After intubation, animals were ventilated with room air with the use of a mechanical ventilator (Harvard Apparatus, Dover, MA). Tidal volume was set to 15 ml/kg, and end-tidal CO2 partial pressure (P ET CO2), measured with an infrared analyzer (Ohmeda, Miami, FL), was kept in a range between 35 and 40 Torr by adjusting respiratory frequency. A femoral artery and vein were dissected, and catheters were introduced for

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measurement of arterial blood pressure and constant infusion of anesthetic, respectively. An additional cubital vein catheter was placed to allow a separate site for drug administration. Tracheal smooth muscle tone was obtained from pressure changes in the saline-filled cuff of an endotracheal tube placed at the level of the 12th to 15th tracheal cartilaginous rings, ~12 cm below the cricoid cartilage, and connected to a solid-state pressure transducer (Ohmeda). Body temperature of the dogs was continuously monitored and regulated via heating pads (Gaymar, Orchard Park, NY). Arterial blood samples were taken for measurement of arterial PO2, PCO2, and pH (model ABL-30, Radiometer, Copenhagen, Denmark). Metabolic acidosis was corrected with slow intravenous infusion of sodium bicarbonate. Three experimental protocols were employed.

Protocol I (n = 8): Electrical stimulation of pararecurrent and recurrent laryngeal nerves. The dogs were placed in the left recumbent position, and access to the pararecurrent and the recurrent laryngeal nerve was achieved through a lateral incision on the right side of the neck, because it has recently been shown that there is a right-sided dominance to innervation of the canine trachea (2). Both nerves were dissected carefully to avoid injury and additional bleeding. The pararecurrent nerve and the recurrent laryngeal nerve were placed on stimulating electrodes (see Fig. 1) in random order and were electrically stimulated for 30 s (10 mA, 100 Hz, 0.1 ms) before and after sectioning of the ramus anastomoticus and superior laryngeal nerve at point X (Fig. 1). Tracheal cuff pressure was continuously measured. At least 3 min were allowed for recovery between stimulations. Two of the eight dogs used in protocol I were also included in protocol III.

Protocol II (n = 6): Reflex and direct stimulation of superior laryngeal and pararecurrent nerves. With dogs in the supine position, the superior laryngeal nerve and the pararecurrent and the recurrent laryngeal nerves on both sides of the neck were dissected through a midline incision. Special care was taken to visualize the anatomic junction of the pararecurrent and the recurrent laryngeal nerves clearly (see Fig. 1). To elicit reflex bronchoconstriction, sodium cyanide (NaCN, 100 µg/kg iv) was administered before and after transection of the superior laryngeal nerve at point X (Fig. 1). Tracheal cuff pressure was measured continuously. Injections were done in duplicate with 5-min intervals allowed for recovery. In addition, the pararecurrent nerve and the superior laryngeal nerve were electrically stimulated for 30 s (10 mA, 100 Hz, 0.1 ms) before and after sectioning of the ramus anastomoticus at point X (Fig. 1).

Protocol III (n = 7): Pararecurrent nerve recordings. After the initial surgery described in protocol I was completed, the pararecurrent nerve was cut close to its entrance into the thorax, and a 2-cm-long desheathed portion was placed on bipolar electrodes. Small branches of the pararecurrent nerve were isolated close to the wall of the trachea (see Fig. 1) and desheathed on a mirrored platform. After desheathing, a single branch was placed on a unipolar electrode for recording nerve action potentials. Simultaneous recordings were made from the phrenic nerve and a branch of the pararecurrent nerve under normal conditions, during administration of NaCN (100 µg/kg), and under hypercarbic conditions produced by the addition of CO2 to inspired air.

Data analysis. Blood pressure, tracheal cuff pressure, PETCO2, and nerve action potentials were continuously recorded and stored on microcomputer (Apple G3 Power PC) using a MacLab data-acquisition system (AD Instruments, Castle Hill, NSW, Australia). Raw nerve activity was amplified 1,000 times by using a battery-operated preamplifier and was then filtered between 100 and 3,000 Hz. After further amplification (10–40 times), nerve action potentials were transferred to computer at a sampling rate of 10 kHz, displayed on an oscilloscope, and monitored audibly on a loudspeaker. Data were analyzed off-line using MacLab software. Raw nerve activity was calculated as root-mean-square values and expressed relative to the baseline activity.

Statistical analysis. To examine the response to nerve stimulation in protocol I, tracheal cuff pressure was analyzed by using two-way repeated-measures ANOVA. In protocol II, the effect of NaCN was tested with a two-way repeated-measures ANOVA. Three-way repeated-measures ANOVA was employed to analyze increases in tracheal cuff pressure to electrical stimulation of the right and left pararecurrent and superior laryngeal nerves. The pararecurrent nerve activity response to administration of NaCN or increase in PETCO2 in protocol III was analyzed by using a one-way repeated-measures ANOVA. Where significant F ratios were found, Tukey’s post hoc test was performed. Data are expressed as means ± SE. The level of statistical significance was set at P < 0.05.

RESULTS

Protocol I. Figure 2 is an example of the typical response to stimulation of the pararecurrent nerve and the recurrent laryngeal nerve. Stimulation of the pararecurrent nerve elicited a substantial increase in tracheal cuff pressure, whereas stimulation of the recurrent laryngeal nerve had little effect. It can also be

Fig. 1. Schematic representation showing anatomic relations among the nerves of interest. Note that the recurrent laryngeal and pararecurrent nerves have separate origins from the vagal trunk and that there are numerous small branches sprouting from the pararecurrent but not the recurrent laryngeal nerve. Electrical stimulations were performed at points S, and the ramus anastomoticus and superior laryngeal nerve (SLN) were sectioned at points X. Recordings were made from fine branches of the pararecurrent nerve. Dashed lines are used to show nerves coursing within the larynx.
seen that stimulation of the two nerves did not cause changes in blood pressure and heart rate. In the group of eight dogs, stimulation of the pararecurrent nerve and the recurrent laryngeal nerve increased tracheal cuff pressure by $21.0 \pm 3.2$ and $1.3 \pm 0.7 \text{cmH}_2\text{O}$, respectively (Fig. 3).

Protocol II. Figure 4 shows summary data for increases in tracheal cuff pressure in response to electrical stimulation of the pararecurrent nerve and the superior laryngeal nerve before and after the ramus anastomoticus was transected. Stimulation of the superior laryngeal nerve failed to elicit an increase in tracheal cuff pressure after the anastomosis was sectioned between the superior laryngeal and pararecurrent nerves. In contrast, transecting the ramus anastomoticus did not alter the bronchoconstrictor response to stimulation of the pararecurrent nerve. Also shown in Fig. 4 is the fact that stimulation of either the pararecurrent nerve or superior laryngeal nerve on the right side elicited significantly greater increases in tracheal cuff pressure than stimulation of the corresponding nerve on the left side. Peripheral chemoreceptor stimulation with NaCN elicited significant increases in tracheal cuff pressure in intact dogs (Table 1). The data in Table 1 show further that the reflex bronchoconstrictor response to NaCN was unaltered by sectioning of the superior laryngeal nerve.

Protocol III. The raw tracing shown in Fig. 5 depicts the characteristic response to arterial chemoreceptor stimulation with intravenous administration of 100 $\mu$g/kg NaCN. Injection of the bolus of NaCN produced marked increases in arterial pressure, tracheal cuff pressure, and pararecurrent nerve activity. These data are summarized in Fig. 6. The pararecurrent nerve activity increased by $208 \pm 51\%$, which was accompanied by a $14.4 \pm 3.5 \text{cmH}_2\text{O}$ elevation in tracheal cuff pressure. Similar results were obtained when CO$_2$ was added to the inspired air. Figure 7 shows that pararecurrent nerve activity increased $49 \pm 19$ and $101 \pm 33\%$ above baseline after elevation in PET$_{CO_2}$ to 50 and 60 Torr, respectively. Corresponding elevations in tracheal cuff pressures were $8.4 \pm 3.6$ and $11.3 \pm 2.9 \text{cmH}_2\text{O}$.

In addition to these findings, in three dogs we were able to further dissect a branch of the pararecurrent nerve and perform recordings on a single-fiber or few-fiber preparation. The results show that baseline firing frequencies of individual preganglionic parasympathetic fibers averaged $2.6 \pm 1.2$ Hz at a PET$_{CO_2}$ of $\sim 40$ Torr. After administration of 100 $\mu$g/kg NaCN, firing frequency increased to $11.6 \pm 1.6$ Hz at peak response.

**DISCUSSION**

The primary results of this study are as follows: 1) stimulation of the pararecurrent nerve, but not the recurrent laryngeal nerve, produced tracheal constriction; 2) although electrical stimulation of the superior laryngeal nerve is capable of producing tracheal constriction via its connection to the pararecurrent nerve, this pathway is not essential for reflex constriction of the caudal cervical trachea; 3) stimuli that produce tracheal constriction increased pararecurrent nerve activity. From these findings we conclude that parasympathetic fibers reach the smooth muscle of the canine trachea via the pararecurrent nerve.

In humans, there is no apparent evidence for the existence of the pararecurrent nerve; thus parasympathetic innervation of the airways is dependent on the vagus nerve and the recurrent laryngeal nerve. In dogs, there is limited physiological evidence to support Lemere's original conclusions (5, 6) about the importance of the pararecurrent nerve in the control of tracheal smooth muscle tone. Brown et al. (1) examined the innervation of the trachea by stimulation of various parasympathetic motor nerves, but there are several serious concerns with their experimental design. First, they were not able to characterize the pararecurrent nerve as a clearly separate structure; therefore, they stimulated the whole vagus nerve with the recurrent laryngeal nerve cut. Second, they may have inadvertently damaged the fine branches of the pararecurrent nerve by inserting a low-cervical tracheostomy and

**Fig. 2.** Original tracings from an individual dog of arterial blood pressure and tracheal cuff pressure response to pararecurrent nerve and recurrent laryngeal nerve stimulation. Note the marked response in tracheal cuff pressure during stimulation of pararecurrent nerve that was almost completely absent during stimulation of recurrent laryngeal nerve.

**Fig. 3.** Summary of the tracheal cuff pressure response to electrical stimulation of pararecurrent nerve and recurrent laryngeal nerve ($n = 8$). *Significantly different from baseline, $P < 0.01$.
preparing discrete segments for tension measurement. Most importantly, Brown et al. ignored the anastomosis between the pararecurrent nerve and the superior laryngeal nerve and assumed that the superior laryngeal nerve contributed independently to tracheal constriction. That probably led them to underestimate the contribution of the pararecurrent nerve.

As seen in Fig. 1, the pararecurrent nerve is a clearly separate structure. It courses in close adherence to the tracheal wall, whereas the recurrent laryngeal nerve is positioned more freely between the vagus nerve and trachea. In our experiments, for recording purposes, a few branches of the pararecurrent nerve were carefully dissected free as they entered the tracheal wall, and tracheal smooth muscle tension was monitored from an intact trachea. Thus there was minimal disruption of the tracheal innervation. One of the important advancements in the present experiments is direct recording of parasympathetic activity in the pararecurrent nerve. The high correlation between changes in pararecurrent nerve activity and tracheal tone coupled with the ability to elicit tracheal constriction by electrical stimulation of the pararecurrent nerve indicate that this is the pathway for parasympathetic nerves to tracheal smooth muscle.

Table 1. Response of tracheal smooth muscle to an intravenous administration of 100 μg/kg sodium cyanide before and after cutting of the superior laryngeal nerve

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<tr>
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<th>Superior Laryngeal Nerve Intact</th>
<th>Superior Laryngeal Nerve Cut</th>
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<td>Baseline cuff pressure, cmH₂O</td>
<td>14.2 ± 0.7</td>
<td>13.9 ± 0.8</td>
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<tr>
<td>Peak response, cmH₂O</td>
<td>36.4 ± 4.1*</td>
<td>36.0 ± 2.8*</td>
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Values are means ± SE; n = 6 dogs. *Significantly different from baseline, P < 0.01.
On the basis of results obtained with electrical stimulation of the superior laryngeal nerve, Brown et al. (1) concluded that the superior laryngeal nerve contributed fibers to both the cranial and caudal cervical trachea. Although the present data confirm that electrical stimulation of the superior laryngeal nerve produces tracheal constriction, transecting the ramus anastomoticus abolishes this response; therefore, it must be concluded that the superior laryngeal nerve contributes parasympathetic fibers to the caudal cervical trachea via its connection to the pararecurrent nerve. Preliminary data in two additional dogs showed that transection of the ramus anastomoticus also abolished constriction of the cranial cervical trachea in response to stimulation of the superior laryngeal nerve. Thus it is evident that the superior laryngeal nerve does not provide independent innervation of the trachea and the pararecurrent nerve is the final pathway for parasympathetic innervation of the canine trachea. Data presented in Table 1 show that the superior laryngeal pathway to the pararecurrent nerve is not essential for reflex constriction of the caudal cervical trachea, although additional pilot data suggest that this pathway may contribute to reflex constriction of the cranial cervical trachea.

There is still a lack of understanding about the complex innervation of the airways and the role that central parasympathetic neurons play in regulation of the airway tone and mucous secretion. Mitchell et al. (8) recorded in vivo intracellular potentials from cat tracheal parasympathetic ganglion cells and traced their axonal projections. They found two types of cells, which were distinguished by their size, location, and projection and were active in a different phase of the respiratory cycle. Clusters of small cells, located in the posterolateral tracheal adventitia, projected to the mucous glands and were active mainly in expiration. A second type of cells had a much larger diameter, located in close apposition to the trachealis muscle, and fired in inspiration. The firing pattern of the fibers from which we recorded exhibited a prominent inhibition during lung inflation. On the basis of this observation, we believe that the recordings in protocol III were from axons directed to tracheal smooth muscle. It is not known whether there is differential control of parasympathetic activity to mucous glands and smooth muscle, although stimulation of pulmonary stretch receptors inhibits parasympathetic efferents to smooth muscle (11) but is not believed to have any effect on efferents to mucous glands (12).

Cell bodies of parasympathetic neurons projecting to tracheal ganglion cells are believed to be located in the
near vicinity of nucleus ambiguus or within the nucleus itself (3, 4, 7). Although immunohistochemical tracing studies have been very useful for initial demonstration of the location of preganglionic cell bodies of parasympathetic neurons, such techniques cannot be applied to locate these neurons for electrophysiological studies. Identification of cell bodies is usually accomplished by electrical stimulation of their axons. Stimulation of the vagus nerve is not selective for parasympathetic nerves to the airways, because the vagus nerves innervate multiple thoracic and abdominal organs. Although the pararecurrent nerve innervates both the trachea and esophagus, it is feasible to separately distinguish tracheal and esophageal branches of the pararecurrent nerve. Based on our results, tracheal branches of the pararecurrent nerve are the best choice for identification of parasympathetic neurons projecting to the trachea. An interesting observation from separate stimulation of right and left superior laryngeal and pararecurrent nerves (Fig. 4) was that there were significantly greater increases in tracheal smooth muscle tone produced by stimulation on the right side. These findings support the recent data (2) showing the right-sided predominance to innervation of the canine trachea and should be taken into account in the design of experiments investigating the central pathways for parasympathetic innervation to the airways.

In that regard, the dog model is a particularly appropriate model for the study of parasympathetic control of the airways because of the absence of nonadrenergic, noncholinergic mechanisms (9). The presence of a discrete parasympathetic pathway in the pararecurrent nerve, together with a method for simple and precise assessment of tracheal tone employing the saline-filled endotracheal cuff, makes this model even more useful. Furthermore, the separate pararecurrent nerve should make it possible to denervate the trachea selectively by sectioning the tracheal branches of the pararecurrent nerve, leaving vagal innervation to other organs intact. Thus the canine preparation is ideal for the study of unanswered questions regarding parasympathetic innervation of the airways, a topic about which our knowledge is incomplete.

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