Chemical profile of vagal preganglionic motor cells innervating the airways in ferrets: the absence of noncholinergic neurons

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Kc, Prabha, Catherine A. Mayer, and Musa A. Haxhiu. Chemical profile of vagal preganglionic motor cells innervating the airways in ferrets: the absence of noncholinergic neurons. J Appl Physiol 97: 1508–1517, 2004; 10.1152/japplphysiol.00282.2004.—In ferrets, we investigated the presence of choline acetyltransferase (ChAT), vasoactive intestinal peptide (VIP), and markers for nitric oxide synthase (NOS) in preganglionic parasympathetic neurons innervating extrathoracic trachea and intrapulmonary airways. Cholera toxin β-subunit, a retrograde axonal transganglionic tracer, was used to identify airway-related vagal preganglionic neurons. Double-labeling immunohistochemistry and confocal microscopy were employed to characterize the chemical nature of identified airway-related vagal preganglionic neurons at a single cell level. Physiological experiments were performed to determine whether activation of the VIP and ChAT coexpressing vagal preganglionic neurons plays a role in relaxation of precontracted airway smooth muscle tone after muscarinic receptor blockade. The results showed that 1) all identified vagal preganglionic neurons innervating extrathoracic and intrapulmonary airways are acetylcholine-producing cells, 2) cholinergic neurons innervating the airways coexpress ChAT and VIP but do not contain NOS, and 3) chemical stimulation of the rostral nucleus ambiguus had no significant effect on precontracted airway smooth muscle tone after muscarinic receptor blockade. These studies indicate that vagal preganglionic neurons are cholinergic in nature and coexpress VIP but do not contain NOS; their stimulation increases cholinergic outflow, without activation of inhibitory nonadrenergic, noncholinergic ganglionic neurons, stimulation of which induces airway smooth muscle relaxation. Furthermore, these studies do not support the possibility of direct inhibitory innervation of airway smooth muscle by vagal preganglionic fibers that contain VIP.

Central nervous system control of the airways; parasympathetic preganglionic neurons; choline acetyl transferase; vasoactive intestinal peptide; nitric oxide synthase; nucleus ambiguus; airway constriction; airway dilation

IN MANY SPECIES, INCLUDING HUMANS, airway smooth muscle (ASM) tone critically relies on the balance between central excitatory cholinergic control (3, 19, 33, 49) and the inhibitory nonadrenergic, noncholinergic (iNANC) influences (2, 12, 17, 47, 56). Activation of cholinergic preganglionic neurons produces airway constriction, submucosal gland secretion and increased blood flow using acetylcholine as a neurotransmitter (23, 25, 26). In the presence of cholinergic and sympathetic blockade, stimulation of vagal nerves, either by electrical current or via neural reflex pathways, elicits relaxation of preconstricted ASM cells via activation of iNANC pathways (2, 12, 17, 56). Recent studies indicate that the iNANC system employs vasoactive intestinal peptide (VIP) (7, 16, 22, 38, 47, 57), nitric oxide (NO) (4, 30, 36, 44, 51), or both (6, 14–16, 64) to elicit ASM relaxation. However, the preganglionic origin of neurons containing these relaxing molecules has not been established.

The preganglionic motor innervation of the airways arises from the nucleus ambiguus and from the most rostral part of the dorsal motor nucleus of the vagus (DMV) (21, 23, 27, 29, 34). Inputs from these preganglionic neurons are carried by the vagal fibers that are thought to synapse with airway ganglionic cells, which in turn modify and distribute central parasympathetic outflow to the effector organs of the airways and lungs (54, 59). However, this concept does not exclude the possibility that some medullary vagal preganglionic motoneurons may provide direct innervation to ASM, secretory glands, or vascular effector organs in the airways. The results of the present and the previous studies (21, 55) clearly document that considerable labeling of vagal preganglionic neurons, preferentially within the rostral nucleus ambiguus region (rNA), occurs after injections of cholera toxin β (CT-β), FluoroGold, or pseudorabies virus into the most distal lung units where intrinsic ganglia are relatively rare. Furthermore, other effector organs, such as the ciliary muscle, receive a substantial proportion of parasympathetic nerves directly from midbrain neurons, bypassing the ciliary ganglion (61).

Physiological studies have shown that the airway vagal preganglionic neurons (AVPNs) within the rNA exert greater effects on ASM tone, submucosal gland secretion, and blood flow, using acetylcholine as a neurotransmitter (23, 25, 26). However, it is not known whether a subset of preganglionic motor neurons innervating the airways contain VIP and/or NO synthase (NOS), an enzyme involved in NO generation, which might modulate the effects acetylcholine. Furthermore, it is not established whether activation of the AVPNs within the rNA, in the presence of cholinergic and adrenergic blockade, produces relaxation of airway muscle constricted with 5-hydroxytryptamine via activation of intrinsic iNANC ganglionic neurons that innervate the ASM.

Therefore, the purpose of the present study was twofold: 1) to characterize the chemical nature of AVPNs and 2) to determine whether activation of AVPNs within the rNA play a role in regulation of elevated ASM tone in the presence of muscarinic and β-adrenergic receptor blockade.

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The results showed for the first time that AVPNs innervating extrathoracic and intrapulmonary airways are cholinergic in nature and express VIP traits but not NOS. Furthermore, stimulation of AVPNs within the rNA increases cholinergic outflow to the airways without activating iNANC ganglionic neurons, which induce ASM relaxation of precontracted airways, in the presence of the muscarinic and β-adrenergic receptor blockade. In addition, these studies do not support the possibility of direct inhibitory innervation of ASM by vagal preganglionic fibers that contain VIP.

**METHODS**

**Animals.** Experimental protocols were approved by the Case Medical School Institutional Animal Care and Use Committee. To minimize eventual physiological effects of hormonal changes, all experiments were performed in male European ferrets, Mustella putorius furo (700–970 g). In the present study, 12 ferrets were used for neuroanatomic experiments, and 8 additional subjects were required for physiological studies.

**Neuroanatomic studies.** Under pentobarbital anesthesia (50 mg/kg ip), the tracheas of six ferrets were injected with CT-β. CT-β was injected along the dorsal tracheal wall, below the third intercartilagineous space. In another group of six animals, five separate 1-μl injections of 0.1% CT-β were made into the lung parenchyma of the right upper lobe by a 10-μl Hamilton syringe, as previously described (21, 29). After 5–7 days, the animals were anesthetized with pentobarbital (50 mg/kg ip), mechanically ventilated with oxygen, and perfused through the left ventricle with 0.1 M sodium PBS, pH 7.4, containing 10,000 U of heparin. This was subsequently followed by a 4% paraformaldehyde in 0.1 M sodium phosphate buffer. The brains were removed and stored in the same fixative for 8–12 h. A series of transverse 40-μm sections were then cut from the level of the spinomedullary junction to the rostral border of the pons using a Bright OTF cryostat (Hacker Instruments and Industries).

**CT-β immunohistochemistry.** The immunohistochemical procedures for staining tissue sections for visualization of CT-β labeled neurons that innervate AVPNs were reported in previous studies (28, 29). Briefly, free-floating sections were washed in PBS containing 0.3% Triton-X. A one-in-five series of tissue sections were exposed for 30 min to a PBS-Triton solution containing 1% BSA to block nonspecific binding sites. After a further wash in PBS, the sections were placed overnight at 4°C in a blocking solution containing rabbit anti-CT-β polyclonal antibody solution (1:20,000; List Biological Laboratories). The sections were washed in PBS-Triton buffer and transferred to secondary antibody [1:200 dilution of donkey anti-rabbit IgG conjugated with Alexa Fluor 488 FITC (Molecular Probes, Eugene, OR) or with Alexa Fluoro 594 (Texas red; Molecular Probes)].

**Choline acetyltransferase immunohistochemistry.** Sequential immunofluorescent detection of the choline acetyltransferase (ChAT) was performed after the CT-β labeling protocol was completed. Briefly, the sections were washed with PBS and incubated for 16 h at 4°C in a solution containing a 1:300 dilution of goat anti-ChAT antibody (Chemicon International, Temecula, CA). After washing in PBS, a 1:200 dilution of secondary antibody (donkey anti-goat IgG), conjugated with Alexa Fluoro 594 (Texas red; Molecular Probes), was applied to tissue sections for 2 h. The rinsed sections were mounted on gelatin/alum-coated glass slides and coverslipped using a drop of VectaShield (Vector Laboratories, Burlingame, CA).

**VIP immunohistochemistry.** In these separate experiments, for CT-β labeling, goat-anti CT-β primary antibody (List Biological Laboratories) was employed, and donkey anti-goat IgG conjugated with Alexa Fluoro 594 (Texas red; Molecular Probes) was used as a secondary antibody in the same dilutions as above described. Sections were subsequently studied for expression of VIP. After washing, they were incubated in a solution containing a 1:2,000 dilution of the rabbit anti-VIP polyclonal antibody (ICN, Costa Mesa, CA). VIP was visualized using a secondary antibody (1:200 donkey anti-rabbit IgG) conjugated with Alexa Fluoro 488 FITC (Molecular Probes). The sections were washed, mounted, and coverslipped.

**NOS histochemistry and immunohistochemistry.** In the present study, nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry was used to determine whether vagal premotor neurons innervating the airways express NOS (24, 60). In these experiments, we first completed the NOS histochemistry protocol and subsequently the CT-β labeling procedure. After washing, tissue sections were incubated in 0.1 M phosphate buffer solution (pH 7.4) containing 1 mM NADPH, 2 mM nitro blue tetrazolium (Sigma Chemical, St. Louis, MO), and 0.3% Triton X-100. The NADPH-diaphorase reaction took place at 37°C for 30–180 min and was then terminated by washing the sections in phosphate buffer for 3 × 10 min each. After washings, CT-β labeling was obtained as described above. NOS immunohistochemistry complemented the NADPH-diaphorase histochemistry. In these experiments, subsequent to CT-β labeling, sections were incubated in a solution containing rabbit anti-neuronal NOS antibody (1:200; Chemicon International, Temecula, CA).

**Control experiments.** Control immunohistochemistry experiments were done to define whether the primary or the secondary antibodies produced false-positive results. Using multiple combinations of primary and secondary antibodies in which a single immunoprobe was omitted. The omission of primary or secondary antibodies, or a VIP preabsorption test, resulted in the absence of immunolabeling, demonstrating that no false-positive results were obtained with these reagents. In addition, NOS expression was not observed when the NADPH substrate was omitted from the reaction mixture.

**Fluorescent and laser scanning confocal microscopy.** Immunostained tissue sections were viewed with fluorescence (Olympus AX70, Olympus America) and laser-scanning confocal microscopes (Olympus BX61, Olympus America) that are equipped with filter systems to observe the Texas red and green FITC fluorescence. Data obtained with confocal microscopy are presented due to the higher sensitivity and specificity of this technique in detecting two fluorescent labels in a single neuron within relatively thick sections. With both methods, immunoprecipitates indicating CT-β, ChAT, or VIP traits were confined to the cytoplasm. Digital images of the exact same sites for CT-β and ChAT, CT-β and VIP, or CT-β and NOS were captured in medullary subregions that contained CT-β immunoreactivity. For each trait, the intensity of the signal for immunolabeled neurons and the intensity of the background signal were measured using Sigma Scan Pro image analysis software (SPSS, Chicago, IL). Only cells that had an intensity twofold above background and were clearly delineated in individual and merged images were counted. Fluorescein (green) and Texas red (red) signals were acquired from the same area of the section, digitized, and stored as tiff files. The software produced overlay (superimposed) images in which the overlap of the red and green signals generated yellow, thereby indicating the degree to which the staining patterns arising from the different antibodies were codistributed.

For each ferret, every doubly labeled section in all series was examined to identify retrogradely labeled neurons and to determine the presence of ChAT, VIP, or NOS in those neurons innervating the airways. The number of retrogradely labeled neurons in each animal was then determined. The spatial separation provided between the samples clearly prevented duplicate counts of the same neurons in selected samples through the neuropil. The reported data are averages of all retrogradely labeled neurons observed on injection of tracer into extrathoracic trachea or intrapulmonary airways.

For detection of NADPH-diaphorase reaction product, a marker for NOS, slides were viewed alternately between bright-field and fluorescence optics. The contrasting immunoprecipitates were readily distinguishable.
Physiological experiments. These experiments were aimed to determine whether activation of AVPNs within the rNA play a role in the regulation of elevated ASM tone in the presence of muscarinic and β-adrenergic receptor blockade. We postulated that chemical activation of AVPNs will cause airway relaxation of precontracted ASM via activation of the intrinsic noncholinergic, nonadrenergic inhibitory ganglionic cells that innervate smooth muscle. To test this hypothesis, eight ferrets were anesthetized with α-chloralose (70 mg/kg ip), and a tracheostomy tube was inserted through the tracheal window placed in the caudal portion of the cervical trachea and connected to a Harvard ventilator. Animals were mechanically ventilated with 100% O2 at a constant volume of 8–10 ml/kg delivered at a frequency of 40–50 breaths/min. Body temperature was continuously monitored through an esophageal probe and maintained at 38–39°C by means of a heating pad. In two of the eight animals, cervical spinal anatomy was performed at the C7 level to eliminate effects of changes in sympathetic outflow on airway responses to rNA stimulation.

After instrumentation, ferrets were placed in a prone position in a head-out plethysmograph, and the head was fixed in a stereotaxic apparatus, with the upper incisor bar 12 mm below the level of the interaural line. A latex collar was fastened to the proximal opening of the plethysmograph to ensure that an adequate seal was achieved, and airway pressure was measured by attaching a pressure transducer to a side port of the tracheal cannula. Flow was measured by placing a pneumotachograph in the distal part of the body box. These signals were used to obtain total lung resistance (RL) by using Buxco software (Buxco Electronics, Troy, NY). The values of RL obtained in this study are presented as means ± SE.

For chemical stimulation of the AVPNs located within the rNA region, the head was fixed in a stereotaxic apparatus, the occipital bone was removed, and the atlanto-occipital membrane was opened. The calamus scriptorius was visualized by incising the overlying dura; this landmark served as a stereotaxic zero for rostrocaudal and lateral coordinates, and its surface was used as the dorsoventral zero. Ferrets were paralyzed with gallamine triethiodide (4 mg/kg iv) to reduce the transmission of cholinergic outflow to the airways induced by pharmacological agents. Flow was measured by placing a pneumotachograph in the distal part of the body box. These signals were used to obtain total lung resistance (RL) by using Buxco software (Buxco Electronics, Troy, NY). The values of RL obtained in this study are presented as means ± SE.

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in the perikaryon of cholinergic cells, and it was observed in retrogradely labeled AVPNs within the rNA and rostral DMV, after CT-β injection into the wall of the extrathoracic trachea or lung parenchyma. This is clearly observable in the overlay images (Figs. 2–4) characterized by the yellowish perikaryon due to the overlap of CT-β-specific (FITC, green) and ChAT-specific (Texas red, red) staining. There were no CT-β-labeled neurons that did not express ChAT; however, there were a number of ChAT-positive but CT-β-negative neurons (Figs. 2 and 3). In these double-labeling experiments, no apparent cross-reactivity of the secondary antibodies was observed (data not shown).

Colocalization of VIP and CT-β. The specific VIP-immunoreactive signal in sections of the medulla oblongata was of relatively lower intensity than that of CT-β or ChAT. However, as can be seen in Fig. 5, the soma could be clearly determined by confocal microscopy. VIP-like immunoreactivity was completely abolished after VIP preabsorption test, as shown in Fig. 5D. All CT-β-labeled neurons that expressed ChAT also expressed VIP. However, not all neurons that coexpressed ChAT and VIP contained the CT-β tracer.

NOS expression. To determine whether AVPNs produce NO, we combined NADPH diaphorase histochemistry and NOS immunohistochemistry with retrograde tracing procedures. Data analysis showed that, within the rNA and dorsal vagal complex regions, retrogradely labeled neurons failed to express a positive NADPH diaphorase reaction. However, strongly positive NADPH diaphorase neurons were observed in other medullary regions, including the ventral portion of the gigantocellular nucleus (Fig. 6). Furthermore, NOS-positive fibers were observed within the rNA region. Similar results were obtained in the experiments where the antibody to NOS was employed (data not shown).

Fig. 1. A: higher power image showing retrogradely labeled airway-related vagal preganglionic neurons (AVPNs) within the rNA 5 days after cholera toxin β-subunit (CT-β) injections into the tracheal wall. B: schematic presentation of a coronal section of the ferret medulla oblongata indicating localization the rNA region. DMV, dorsal motor nucleus of vagus; LV, lateral vestibular nucleus; MeV, medial vestibular nucleus. Scale bar = 100 μm (A) and 1 mm (B).

Fig. 2. A: confocal photomicrographs showing retrogradely labeled AVPNs within the rNA 5 days after CT-β injections into the tracheal wall. B: same section immunostained for choline acetyltransferase (ChAT). C: a higher power of the merged (M) image of CT-β and ChAT traits of the region marked by a quadrangle in A. Arrow indicates a representative neuron that is immunolabeled with CT-β and ChAT. *ChAT-positive neuron that does not contain CT-β. Scale bar = 100 μm for A and B and 40 μm for C. D: quantitative analysis of the ChAT-positive neurons within rNA. AVPNs that were positive for CT-β alone, and number of AVPNs that contained both CT-β and ChAT. Note that all retrogradely labeled neurons express ChAT.
Fig. 3. A: confocal photomicrographs showing retrogradely labeled AVPNs within the rNA 5 days after CT-β injections into the lung. B: same section immunostained for ChAT. C: higher power of the M image of CT-β and ChAT traits of the region marked by a quadrangle in A. Arrow indicates a representative neuron that is immunolabeled with CT-β and ChAT. *ChAT-positive neuron that does not contain CT-β. Scale bar = 50 μm for A and B and 35 μm for C. D: quantitative analysis of the ChAT-positive neurons within rNA, AVPNs that were positive for CT-β alone, and the number of AVPNs that contained both CT-β and ChAT. Note that all retrogradely labeled neurons express ChAT.

Fig. 4. A: confocal photomicrographs showing retrogradely labeled AVPNs within the rostral dorsal motor nucleus of the vagus (DMV) 5 days after CT-β injections into the extrathoracic trachea. B: same section immunostained for ChAT. C: M image of CT-β and ChAT traits. Arrow indicates a representative neuron that is immunolabeled with CT-β and ChAT. *ChAT-positive neuron that does not contain CT-β. D: schematic presentation of a coronal section of the ferret medulla oblongata indicating localization of the DMV region. Scale bar = 20 μm for A–C and 1 mm for D. As in rNA, all CT-β-labeled neurons expressed ChAT-like immunoreactivity.
Physiological experiments. Stimulation of AVPNs by glutamate before blockade of muscarinic receptors induced an increase in ASM tone that was manifested with an increase in Ptseg and RL. Ptseg increased from 8.8 \( \pm \) 2 to 18.9 \( \pm \) 1.8 cmH\(_2\)O (\( P = 0.001 \)) and RL from 0.09 \( \pm \) 0.016 to 0.110 \( \pm \) 0.019 cmH\(_2\)O\( \cdot \)ml\(^{-1}\)s (\( P = 0.029 \)). To reveal the relaxation components of vagal stimulation, we used antagonists of muscarinic cholinergic receptors and \(-\)adrenergic receptors to block the excitatory responses to acetylcholine and relaxing effects of catecholamines on ASM, respectively. Before intravenous administration of atropine methyl nitrate, ventilatory rate was decreased to increase cholinergic outflow to the airways. Administration of atropine methyl nitrate abolished cholinergic outflow to the airways. Ptseg decreased from 25 \( \pm \) 2 to 9 \( \pm \) 1 cmH\(_2\)O (\( P = <0.001 \)) and Rt. from 0.111 \( \pm \) 0.008 to 0.0731 \( \pm \) 0.012 cmH\(_2\)O\( \cdot \)ml\(^{-1}\)s (\( P = 0.014 \)). Infusion of serotonin increased Ptseg from 9.1 \( \pm \) 1 to 29.1 \( \pm \) 2 cmH\(_2\)O (\( P = 0.001 \)) and Rt. from 0.0731 \( \pm \) 0.012 to 0.229 \( \pm \) 0.04 cmH\(_2\)O\( \cdot \)ml\(^{-1}\)s (\( P = 0.001 \)). During serotonin infusion, when steady state was reached, stimulation of AVPNs by glutamate had no significant effect on Ptseg or RL; [Ptseg changed from 28.9 \( \pm \) 2 to 29.1 \( \pm \) 2 cmH\(_2\)O (\( P = 0.986 \)) and Rt. from 0.229 \( \pm \) 0.04 to 0.241 \( \pm \) 0.042 cmH\(_2\)O\( \cdot \)ml\(^{-1}\)s (\( P = 0.841 \)); Fig. 7].
Effects of L-glutamate stimulation of rNA on arterial pressure. To record cardiovascular status, we measured arterial pressure and heart rate. In ferrets, after blockade of muscarinic receptors by atropine methylnitrate, systemic infusion of 5-HT increased mean arterial pressure from 108 ± 13 to 134 ± 10 mmHg (P = 0.134) and heart rate from 306 ± 17 to 330 ± 15 beats/min (P = 0.296). L-Glutamate microinjected into rNA caused an increase in mean arterial pressure (from 134 ± 10 to 152 ± 12 mmHg; P = 0.263) and heart rate (from 330 ± 15 to 338 ± 14 beats/min; P = 0.7).

**DISCUSSION**

The results of the present study confirm previous findings, showing that, in the ferret, as in the rat, cat, and dog, parasympathetic preganglionic outflow to the airways arises mainly from the rNA and to lesser extent from the DMV (23, 25, 26, 29).

**Cholinergic traits in AVPNs.** The present results, obtained by combining the retrograde tracer CT-β and immunohistochemistry for ChAT, indicate that AVPNs innervating trachea and the intrapulmonary airways are cholinergic in nature and use acetylcholine as a neurotransmitter. These neurochemical findings uphold physiological studies showing that stimulation of AVPNs or efferent fibers in the trunk of the vagus nerve produces pronounced contraction of the ASM that is solely mediated via cholinergic mechanisms (23, 26, 50).

Conceivably, in the present study, not all vagal preganglionic neurons that innervate tracheobronchial system could be labeled. However, the analyzed sample size in 12 studied ferrets likely represents the actual chemical profile of AVPNs. Furthermore, quantitative analysis of brain tissues processed for immunocytochemistry is relatively imprecise. This is because the quality of immunocytochemical labeling is affected by tissue fixation. Thus, as structure is preserved with strong fixatives, the immunocytochemical signal is often severely degraded. Therefore, the loss of antigenicity and inefficient antibody penetration could affect visualization of neurotransmitters. In this study, all analyzed tissue sections were cut in their entirety, and robust ChAT labeling was observed in regions previously shown to contain ChAT-producing cell bodies (31, 58). Furthermore, control experiments clearly demonstrated the specificity of ChAT staining, indicating that it is highly unlikely that noncholinergic parasympathetic preganglionic neurons innervate the airways.

Similarly, studies in cats have suggested that the vagal cholinergic preganglionic neurons controlling negative chronotropic, dromotropic, and inotropic responses are distributed mainly in the nucleus ambiguus (45). However, in rats, a population of vagal preganglionic cell bodies within the external portion of the nucleus ambiguus that project to the heart do not express cholinergic traits (58). These dissimilarities in neurotransmitter profile of AVPNs in ferrets and cardiac vagal preganglionic cells in rats could be due to species differences or differences in the target sites studied.

In the ferret, within rostral ventrolateral medulla are found catecholaminergic neurons. The AVPNs do not express catecholaminergic traits, but tyrosine hydroxylase-positive axon terminals lie in their close proximity and, when stimulated, release norepinephrine within the rNA region causing withdrawal of cholinergic outflow to the airways (28). Similarly, in cats, tyrosine hydroxylase-immunoreactive terminals innervate nonadrenergic cardiac vagal preganglionic cells, commonly forming axodendritic synapses on these negative inotropic neurons (45).

**VIP traits in AVPNs.** The results of the present study showed that, in ferrets, virtually all vagal preganglionic neurons innervating trachea and intrapulmonary airways are cholinergic in nature and coexpress VIP, indicating that acetylcholine and neuropeptide VIP are coexisting messenger molecules in AVPNs. Contrary to AVPNs, only one-third of all cortical intrinsic cholinergic neurons contain immunoreactivity for VIP (5, 11).

The neuropeptide VIP first isolated from the small intestine in 1970 (57) is synthesized together with peptide histidine isoleucine from a common precursor, preproVIP, and both peptides are highly expressed in the central (43) and peripheral nervous system, including airway ganglionic neurons (14–16, 38, 64). VIP contains 28 amino acid residues, and its primary structure is closely related to pituitary adenylate cyclase-activating polypeptide, and the two peptides share the same receptors (43).

The role of VIP expressed by AVPNs in central regulation of airway functions is not clear. Similar to other neurotransmitters and/or neuromodulators, VIP originating from AVPNs can participate in regulation of ASM tone acting presynaptically or postsynaptically at the level of vagal preganglionic neurons and/or at the synapse with the intrinsic ganglionic cells. It is less likely that VIP coexpressed with ChAT in the AVPNs affects the ASM tone via direct projections, bypassing intrinsic airway ganglia.

Fig. 7. In ferrets, after atropine methylnitrate administration and the restoration of tracheal tone (P strained; top) and lung resistance (RL; bottom) by 5-HT, L-glutamate (L-Glu) injection into the rNA region had no effect on Ptseg or RL. The average Ptseg in a control period (CON) was comparable to that measured after stimulation of the rNA by L-Glu.
It has been demonstrated that released VIP can augment acetylcholine synthesis (39, 41), potentiate acetylcholine actions via enhancement of muscarinic or nicotinic receptor functions (35, 42), augment release of acetylcholine in the hippocampus (46), and increase the strength of glutamatergic neurotransmission (43).

The results of the present study clearly demonstrate that AVPNs that contain VIP are cholinergic and excitatory in nature. Stimulation of bronchopulmonary sensory receptors and activation of glutamatergic pathways (25) causes burst firing of AVPNs (53). Such discharge would be the appropriate stimulus for release of neuropeptide VIP at ganglionic sites that, in turn, might influence airway ganglionic transmission (54, 59) and the final neuronal output. VIP acts on the VIP1–3 receptors (43), activation of which stimulates neurons along the neural axis, including the superior cervical ganglion cells (42). In addition, with the use of electron microscopy, the presence of this peptide in cholinergic nerves was observed (38), suggesting that it could be coreleased with acetylcholine at the synapse with intrinsic ganglionic neurons and may function as a signaling molecule. Therefore, it could be assumed that VIP coreleased with acetylcholine will enhance synaptic efficacy in excitatory airway ganglia, augmenting contraction rather than causing ASM relaxation. However, there are studies indicating that VIP inhibits cholinergic neurotransmission at both ganglionic and postganglionic sites in the guinea pig, dog, and cat isolated trachea, in addition to its postjunctional relaxing effects on ASM cells (6, 7, 22).

Neurochemical studies have clearly demonstrated the presence of relaxant substances in intrinsic airway ganglionic neurons and postganglionic fibers of several mammals, including humans (14–16, 38, 64). In ferrets, intrinsic excitatory ganglionic cells of tracheal plexus that contain ChAT are predominantly located in the longitudinal trunk, whereas VIP, NOS, and substance P-producing neurons are mainly found in the superficial muscular plexus ganglia. Furthermore, the majority of VIP-containing neurons express NOS (14, 15, 64). Hence, stimulation of parasympathetic preganglionic cells may activate both inhibitory and excitatory intrinsic neurons, which provide parallel contractile and relaxant innervation of ASM cells. The excitatory ganglionic neurons use ACh and/or substance P to increase smooth muscle tone and constrict the tracheobronchial system (9, 10, 19, 54, 59), whereas the ganglionic inhibitory neurons employ VIP and/or NO to relax ASM cells and dilate the airways (7, 14–16, 47, 64). Our physiological studies, however, do not suggest that, in the presence of muscarinic and β-adrenergic receptor blockade, stimulation of AVPNs activate a critical number of inhibitory ganglionic pathways that lead to dilation of precontracted airways. Furthermore, these studies do not support the possibility of direct inhibitory innervation of ASM by vagal preganglionic fibers that contain VIP.

Previous studies showed that, in guinea pigs, the airway cholinergic and noncholinergic parasympathetic nerves represent distinct vagal pathways, mediating contractile or relaxant responses (10, 48). Furthermore, there is no evidence for corelease of acetylcholine and putative relaxant neurotransmitters at airway effector sites (19). Recently, it has been shown that VIP/NOS-containing parasympathetic nerves innervating the guinea pig airways originate in the nearby esophagus. Hence, disrupting the tissue between the esophagus and trachea abolishes vagally mediated relaxant responses in the trachea, while preserving cholinergic contractions (10, 48).

**NOS and AVPNs.** The results of the present study showed that retrogradely labeled AVPNs do not express NADPH diaphorase reaction. However, strongly positive NADPH diaphorase neurons were observed in other medullary regions, including the nucleus tractus solitarius and ventral portion of the gigantocellular nucleus that provide afferent innervation to AVPNs (21, 27). In the present study, comparable results were obtained using NADPH-diaphorase staining and NOS immunoreactivity, which indicates that NADPH-diaphorase staining reflects the presence of neuronal NOS (25, 60). These results do not support the suggestion that brain stem vagal preganglionic neurons can be localized by markers for NOS (20).

Contrary to AVPNs, immunohistochemical characterization of cardiac vagal preganglionic neurons in the rat revealed NOS-containing neurons in the DMV (58). Furthermore, brain stem neurons regulating salivary gland function express NOS (63). NOS is present in a portion of vagal preganglionic neurons in the DMV, innervating the low esophageal sphincter, as well as in a subpopulation of vagal motoneurons that project to the gastric fundus, excitation of which evokes low esophageal sphincter and gastric relaxation (1, 32, 37, 62).

**Physiological studies and relevance of present findings.** Stimulation of the rNA, after an increase of ASM tone by systemic infusion of serotonin after muscarinic and adrenergic blockade, was not associated with airway dilation, decrease in tracheal tone, and Rt.. Unlike lower airways, previous studies showed that, in cat and ferret, extrathoracic trachea is not under the control of iNANC pathways (18, 50). Conceivably, reflex relaxation of the lower ASM could be mediated via the DMV. However, this is highly unlikely since stimulation of these preganglionic vagal motoneurons has no effect on bronchomotor tone (23). Hence, the results of the present study suggest that a critical subpopulation of intrinsic inhibitory ganglionic neurons that is needed to elicit relaxation of the precontracted smooth muscle does not receive efferent excitatory innervation originating from AVPNs located in the rNA region. The intrinsic VIPergic and nitrergic neurons may receive local excitatory sensory inputs, triggered by humoral and inflammatory factors, which allow discrete regulation of ASM tone, controlling it from within (13), as is the case with intrinsic excitatory communicating pathways (9).

In summary, the results of the present study strongly suggest that airway-related preganglionic parasympathetic neurons that coexpress ChAT and VIP do not exert measurable stimulatory effects on intrinsic inhibitory ganglionic neurons or direct relaxation of the ASM, bypassing ganglionic cells. AVPNs from the rNA provide cholinergic innervation to intrinsic excitatory ganglionic neurons regulating smooth muscle tone. Therefore, iNANC influences are mediated through distinct humoral and neural pathways.

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REFERENCES

27. Masuri V, Matsumoto Y, Tokito F, Tsuda M, and Fujino M. Effects of vasoactive intestinal polypeptide (VIP) and pituitary adenyly cyclase...


