Catecholaminergic microcircuitry controlling the output of airway-related vagal preganglionic neurons

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Haxhiu, Musa A., Prabha Kc, Burim Neziri, Bryan K. Yamamoto, Donald G. Ferguson, and V. John Massari. Catecholaminergic microcircuitry controlling the output of airway-related vagal preganglionic neurons. J Appl Physiol 94: 1999–2009, 2003. First published January 3, 2003; 10.1152/japplphysiol.01066.2002.—In this study, we have investigated the ultrastructure and function of the catecholaminergic circuitry modulating the output of airway-related vagal preganglionic neurons (AVPNs) in ferrets. Immunoelectron microscopy was employed to characterize the nature of catecholaminergic innervation of AVPN at the ultrastructural level. In addition, immunofluorescence was used to examine the expression of the α2A-adrenergic receptor (α2A-AR) on AVPNs, and norepinephrine release within the rostral nucleus ambiguous (rNA) was measured by using microdialysis. Physiological experiments were performed to determine the effects of stimulation of the noradrenergic locus coeruleus (LC) cell group on airway smooth muscle tone. The results showed that 1) catecholaminergic nerve endings terminate in the vicinity of identified AVPNs but very rarely form axosomatic or axodendritic synapses with AVPNs that innervate the extrathoracic trachea; 2) AVPNs express the α2A-AR; 3) LC stimulation-induced norepinephrine release within the rNA region was associated with airway smooth muscle relaxation; and 4) blockade of α2A-AR on AVPNs diminished the inhibitory effects of LC stimulation on airway smooth muscle tone. It is concluded that a noradrenergic circuit originating within the LC is involved in the regulation of AVPN activity within the rNA, and stimulation of the LC dilates the airways by the release of norepinephrine and activation of α2A-AR expressed by AVPNs, mainly via volume transmission.

PARASYMPATHETIC INNERVATION of the airways arises from the nucleus ambiguus and from the dorsal motor nucleus of the vagus (22, 25, 28). Between these two groups of neurons, the airway vagal preganglionic neurons (AVPNs) within the rostral nucleus ambiguous (rNA) play a greater role in providing cholinergic outflow to the airways (18). Their activity relies on afferent inputs and can be reduced or augmented by projections arising from different sites, including pontine nuclei and monoaminergic cell groups (21, 48). Many of the central neural mechanisms involved in control of airway smooth muscle are likely to apply also to the control of secretory glands and blood flow (6, 19, 25, 51).

The vagal preganglionic motor cells are innervated by a network of brain stem catecholaminergic neurons, in particular norepinephrine-containing cells (17, 21, 36, 49), known to be involved in the regulation of autonomic functions, motor activity, and the sleep-wake- aroused cycle (2, 27, 46, 55). The major noradrenergic inputs to the AVPNs arise from the A5 cell group, the locus coeruleus (LC) and subcoeruleus (17, 21, 49). Their role in central regulation of cholinergic outflow to the airways and the mode of action of released norepinephrine on modulating cholinergic outflow to the airways are not known. However, as in other systems, these effects may be mediated by direct monosynaptic contacts of norepinephrine-containing nerve terminals on AVPN neurons, by nonsynaptic mechanisms via volume transmission, or both (1, 7). Therefore, one of the aims of the present study was to investigate the neuroanatomical substrates of catecholaminergic innervation, by analyzing whether catecholamine-containing terminals make synaptic contacts with identified AVPNs, and, if so, to characterize the magnitude and distribution of these contacts on somata and dendrites.

After vesicular release, norepinephrine acts at targeted sites, eliciting responses that depend on the expression of specific adrenergic receptor subtypes. The adrenergic receptor family is composed of three subfamilies (α1, α2, and β) each containing a minimum of three distinct subtypes. Each subtype is coded by a separate gene and displays characteristic tissue distribution, regulatory properties, and drug specificities (10, 32). As opposed to the α1, and β-adrenergic receptors, activation of the α2-adrenoceptors (α2-ARs) by norepinephrine inhibits neuronal activity (10, 61). We hypothesize that norepinephrine-containing neurons are the potential source of the noradrenergic inhibitory
drive to AVPNs, acting via $\alpha_2$-ARs. This hypothesis is supported by earlier studies, showing that the I1-imidazoline agonist moxonidine suppresses reflex airway constriction by a central mechanism. This effect could be significantly reversed by efaroxan (an I1-imidazoline and $\alpha_2$-AR blocker), suggesting that these receptor classes may be involved in brain stem control of the cholinergic outflow to the airways (20), in parallel to their involvement in central regulation of sympathetic activity and arterial pressure (37). However, there is no information confirming that $\alpha_2$-ARs are expressed by AVPNs.

The $\alpha_2$-ARs are divided into four subtypes, based primarily on radioligand binding characteristics in native tissue homogenates. The $\alpha_2A$-ARs, characterized by relatively high affinity for yohimbine and rauwolscine, are present in lower brain stem neurons, including catecholaminergic and serotonergic cells innervating the spinal cord (5, 16). Furthermore, the $\alpha_2A$-ARs are expressed on glutamatergic nerve terminals, where their activation could inhibit glutamate release and excitatory synaptic transmission (4, 14).

In this study, we tested the hypothesis that activation of noradrenergic cell groups that project to the AVPNs causes release of norepinephrine from nerve terminals, a subset of which make synaptic contacts with AVPNs and their processes, thereby inducing an inhibition of AVPNs via activation of $\alpha_2A$-ARs expressed by these cells. This would lead to withdrawal of cholinergic outflow to the airways and airway smooth muscle relaxation.

**METHODS**

The studies were performed with the use of a total of 29 male European ferrets, *Mustella putorius furo* (650–950 g). Four animals were used for neuroanatomical experiments, six ferrets were employed to examine the expression of $\alpha_2A$-AR on AVPNs within the rNA, eleven ferrets were used for microdialysis studies and measurements of norepinephrine release, and eight ferrets were required for physiological experiments.

**Ultrastructural studies of noradrenergic innervation of AVPNs: electron microscopy.** Under pentobarbital anesthesia (50 mg/kg ip), the trachea of four ferrets was injected with cholora toxin $\beta$ subunit conjugated to horseradish peroxidase (CTb-HRP). As previously described, CTb-HRP was injected along the tracheal wall beginning with the third intercartilaginous space (22). After 4 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 mixture of 2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium phosphate buffer. The brains were removed and stored in the same fixative for 8–12 h. Two series of transverse 40-μm sections were then cut from the level of the spinomedullary junction to the rostral border of thepons by using a vibratome. Free-floating sections were processed to reveal CTb-HRP-labeled cell bodies by a modification of the tungstate stabilized tetramethylbenzidine (TMB) method of Weinberg and van Eyck (60). This protocol results in the formation of a crystalline electron-dense reaction product in TMB-immunoreactive profiles. Processing for light and electron microscopy, photography, sampling, and analysis was performed as earlier described (39, 40).

Briefly, for light microscopy, one series of tissues was mounted on glass slides, dehydrated in ethanol, cleared in xylene, coverslipped with Permount and examined in a Nikon FXA photomicroscope equipped with bright-field, dark-field, and differential interference contrast optics. For electron microscopy, an alternate series of tissues was postfixed in 2% osmium tetroxide in PBS for 1 h at room temperature, dehydrated through a graded series of ethanol and propylene oxide, embedded in epoxy resin between two sheets of plastic (Aclar; Du Pont), and cured at 60°C for 48 h. Embedded tissues were examined in a light microscope, and areas of interest including the nucleus ambiguus were cut out and reembedded in Beem capsules. Serial ultrathin sections of the reembedded tissues were cut on an ultramicrotome (Reichert, Ultracut S) at ~75-nm thickness (silver-gold interference color), collected on copper grids, poststained with uranyl acetate and lead citrate, and examined in a JEOL JEM-1210 transmission electron microscope at 50 kV accelerating voltage.

For quantitative analysis, one 40-μm section that exhibited the best combination of morphological preservation, immunocytochemical labeling, and retrograde transport was used from each of four ferrets. From each 40-μm-thick section, three ultrathin sections were cut and examined. These samples were taken from areas close to both the plastic-tissue interfaces on each side of the section, as well as the center of the tissue.

For each ferret, quantitative data were obtained from a series of three ultrathin sections separated from each other by not less than 18 μm. The spatial separation provided between the samples clearly prevented duplicate counts of the same terminal in our three samples through the neuropi. The total area examined for all four animals was ~6,000 μm². The surface area of these sections was examined systematically at ×8,000 magnification for the presence of the TMB-tungstate crystalline reaction product, which was readily observed in retrogradely labeled AVPNs. The entire circumference of all retrogradely labeled profiles was then photographed at magnifications of 4,000–×20,000 to obtain a record of all potential synaptic contacts. Each thick section contained three to eight retrogradely labeled neurons. However, it was common to find these neurons stratified within the depth of the tissue. As such, not all neurons contained within the tissue were observed within each ultrathin section that was sampled. The reported data are averages of all labeled and unlabeled axodendritic and axosomatic synapses that were observed on retrogradely labeled tracheal AVPNs.

Neuronal profiles were identified on the basis of the previous ultrastructural descriptions (50). Neuronal somata were defined as profiles containing a nucleus. Dendrites contained regular microtubule arrays and were postsynaptic to axon terminals. Axon terminals were defined as profiles
containing numerous small synaptic vesicles with a cross-sectional diameter \(>0.5\ \mu m\). Profiles \(<0.5\ \mu m\) in diameter that contained a few small vesicles but lacked a synaptic junction were considered unmyelinated axons. Profiles wrapped in multiple electron-dense lamellae were considered myelinated axons. Asymmetric synapses were characterized by a wide synaptic cleft and prominent postsynaptic density, whereas symmetric synapses had a narrow synaptic cleft and pre- and postsynaptic densities that were less prominent than those observed in asymmetric synapses (15, 50). Terminals that were in close apposition to retrogradely labeled AVPN profiles, but that did not display a clear synaptic contact in the plane of section examined, were not counted.

\(\alpha_{2AAR}\) immunocytochemistry: laser scanning confocal microscopy. Immunofluorescent detection of the receptors expressed by neurons identified as AVPNs complemented the ultrastructural studies and was performed in another group of six ferrets. Double labeling was used to examine the distribution of the \(\alpha_{2AAR}\) on AVPNs that had been identified by using a retrograde tracer cholera toxin subunit (CTb), as earlier described (24, 35). Briefly, after anesthesia, CTb was microinjected into the wall of the extrathoracic trachea. After five days of survival, ferrets were deeply anesthetized and perfused with 4% paraformaldehyde, and 50-\(\mu m\) frozen sections of the rostral medulla oblongata were cut. Sequential immunohistochemistry was then performed to determine whether \(\alpha_{2AAR}\) were expressed by identified tracheal AVPNs located in the rNA. In the first step, free-floating sections were washed in PBS containing 0.3% Triton-X and then transferred for 30 min to PBS-Triton solution containing 1% bovine serum albumin to block nonspecific binding sites. After a second 30-min wash, the sections were incubated overnight at 4°C in the blocking solution containing a goat anti-\(\alpha_{2AAR}\) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). The sections then were rinsed, incubated with biotinylated donkey anti-goat (1:500) serum, (Jackson Immunotech, West Grove, PA) for 3 h at room temperature, and after a third wash the sections were further processed by use of the standard biotin-avidin-peroxidase kit (Vector, ABC-elite kit, Vector Laboratories, Burlingame, CA). The immunoreaction was visualized by incubating the sections with 0.02% 3,3'-diaminobenzidine containing 0.01% hydrogen peroxide for 1–3 min. The sections were rinsed with PBS and then incubated for 16 h at 4°C in a solution containing a rabbit anti CTb serum (1:20,000; Accurate Chemical and Scientific, New York, NY). The sections were washed in PBS-Triton buffer and transferred to secondary antibody [1:200 dilution of goat anti-rabbit IgG conjugated with Alexa Fluor 594, i.e., Texas Red (TR); Molecular Probes, Eugene, OR]. The rinsed sections were mounted on gelatin/alum-coated glass slides and coverslipped by using a drop of VectaShield (Vector Laboratories). In addition, a second 1-in-5 series of sections was analogously stained for laser scanning confocal microscopy by use of secondary antibodies conjugated with TR (red) for \(\alpha_{2AAR}\) and fluorescein isothiocyanate (FITC, green) for CTb.

Slides were viewed with a fluorescence microscope (Olympus AX70, Olympus America, Melville, NY) equipped with appropriate filter systems to observe the TR fluorescence and bright-field optics to observe expression of \(\alpha_{2AAR}\). Colocalization of \(\alpha_{2AAR}\) protein with the CTb was identified by viewing the sections alternately between bright-field optics and fluorescence optics. The contrasting immunoprecipitates were readily distinguishable. Sections were also examined and digitized, and indirect immunofluorescence images were collected by use of a Leica TCS-SP2 laser scanning confocal microscope. In these experiments, CTb was detected by using a fluorescein-conjugated secondary antibody, and the receptor was detected employing a TR-conjugated secondary antibody. Fluorescein (green) and TR (red) signals were acquired from the same area of the section, digitized, and stored as tiff files. The Leica 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. Specificity controls were obtained by replicating the experimental conditions in the absence of primary antibody.

Microdialysis and HPLC measurements of norepinephrine release. Measurements of norepinephrine release were performed in 11 ferrets that were anesthetized with \(\alpha\)-chloralose (70 mg/kg ip), tracheotomized, and carotid artery and jugular vein cannulated. A tracheostomy tube was inserted through a tracheal window placed in the caudal portion of the cervical trachea and connected to a Harvard ventilator. Animals were subsequently paralyzed (gallamine hydrochloride, 4 mg/kg iv) and mechanically ventilated with 100% oxygen at a constant volume of 7 ml/kg delivered at a frequency of 30–35 breaths/min. Body temperature was continuously monitored through an esophageal probe and maintained at 35–36°C by means of a heating pad.

After instrumentation, ferrets were placed in a prone position in a stereotaxic apparatus. A concentric-shaped microdialysis probe with a tip diameter of 0.21 mm was constructed as previously described (12, 62). The dialysis membrane (Spectrapor, 13,000 molecular weight cutoff) was 1.5 mm in length. The probe was inserted unilaterally into the rostral ventrolateral portion of the medulla oblongata, 3.5 mm rostral to the calamus scriptorius, 3.0 mm lateral to the midline, and 1.5–1.5 mm dorsal to the ventral medullary surface. In this region of the ferret brain stem, we have previously observed clustered AVPNs that project to the extrathoracic trachea (22). After placement of the microdialysis probes, Dulbecco's PBS (containing, in mM, 138 NaCl, 2.7 KCl, 0.5 MgCl\(_2\), 1.5 KH\(_2\)PO\(_4\), 8.1 Na\(_2\)HPO\(_4\), 1.2 CaCl\(_2\), and 0.5 \(d\)-glucose, pH 7.4) was perfused through the probes. The flow rate was maintained at 2.5 \(\mu l/min\) by use of a microinjection pump (Harvard Instruments, South Natick, MA). A minimum of 2 h was allowed for equilibration of the dialysis probes, and then three 20-min baseline samples were collected on ice and immediately analyzed for norepinephrine concentration. In eight ferrets, after collection of three 20-min baseline samples, unilateral stimulation of the LC was then induced by pressure microinjection of glutamate (4 nmol/80 nl per site) by using a glass micropipette with a 40-\(\mu m\) tip diameter placed into the LC, 11 mm rostral to the area postrema, 2.2 mm lateral to the midline, and 5 mm dorsal to ventral surface. Microinjections of glutamate were performed every 5 min for a period of 20 min. In three control ferrets, no stimulation was performed and microdialysates were obtained to measure norepinephrine release at the same time points as in experiments with LC stimulation. Each sample was immediately processed for measurement of the norepinephrine content of the dialysate. At the end of each experiment, 80 nl of 1% fast green dye was injected through the second barrel of the micropipette into the LC, or via the microdialysis probe into the rNA region to permit histological identification of the location of the micropipette or probe. Tissue sections with greatest dye density were considered to be the injection or microperfusion sites. Figure 1 shows regions within the dorsal pons (LC) and rostral ventrolateral medulla (rNA) where interventions were made.

Each dialysate sample (20 \(\mu l\)) was assayed for norepinephrine by HPLC with electrochemical detection. A mobile phase
Fig. 1. Coronal sections of the ferret brain stem indicating sites examined in these studies. A: locus coeruleus and subcoeruleus region (LC) where glutamate was injected (box). B: higher power image of the area indicated by the box in A, showing tyrosine hydroxylase (TH)-positive cells. C: rostral nucleus ambiguous region (rNA) in which the microdialysis probe was placed (box). D: higher power image of the area indicated by the box in C, showing retrogradely labeled airway-related vagal preganglionic neurons (AVPNs) within rNA. 5 days after cholera toxin β subunit (CTb) injections into tracheal wall. Bar = 1 mm (A, C), 100 μm (B), 150 μm (D).

**Physiological experiments.** In a separate series of experiments, ferrets were anesthetized, paralyzed, and mechanically ventilated as for microdialysis and HPLC measurements of norepinephrine release. In these animals, we investigated the effects of LC stimulation on airway smooth muscle tone and the mechanisms involved in airway smooth muscle relaxation induced by LC stimulation. Airway smooth muscle responses evoked by stimulation of the LC and subcoeruleus (the A6 cell group) were measured as changes in tracheal smooth muscle tone. Tracheal smooth muscle tone was assessed indirectly by measuring the changes in pressure (in cmH2O) in a balloon placed in a bypassed rostral segment of the cervical trachea, as previously described (24).

In bypassing the extrathoracic tracheal segment, care was taken not to damage the recurrent and superior laryngeal nerves and the plexus of ganglia on the posterior wall or to interrupt the blood supply. The balloon in the extrathoracic trachea was distended with 0.8-1.2 ml of saline.

Initial measurements were performed to ensure that the efferent transmission of cholinergic outflow to the airways was not affected by the surgery. This was achieved by demonstrating that the reflex responses of tracheal smooth muscle tone to hyperoxic hypercapnia were intact. To determine basal tracheal tone, the pressure in the balloon (P_{t_{es}}) was measured after withdrawal of cholinergic outflow to the airways induced by hyperoxic hypocapnia. The hyperoxic hypercapnia was produced by gradually increasing the rate of the ventilator to lower arterial CO2 and consequently to reduce the tracheal tone to ~10 cmH2O. This value was considered to be basal tracheal tone and was close to that recorded after intravenous administration of atropine, as was previously described in cats (45). After the minimum level of cholinergic activity was established, the rate of the ventilator was decreased to restore airway smooth muscle tone by increasing arterial CO2 pressure (between 37 and 45 Torr). Once a steady state was reached, the LC was stimulated as described above.

The effects of LC stimulation on airway smooth muscle tone were studied in eight ferrets pretreated with propranolol (1 mg/kg iv). In these animals, we determined the potential role of α2A-ARs in mediating airway responses to stimulation of the LC. Changes in airway smooth muscle tone were examined before and after bilateral microperfusion of α2A-AR receptor antagonists: the SK&F 86466 (10 μM, 2.5 μl/min; n = 5) or yohimbine (10 μM, 2.5 μl/min; n = 3), via microdialysis probes that were stereotactically advanced into the rNA. In a control period and 15 min after initiation of microdialysis of drugs, the LC was stimulated by microinjection of 4 nmol of l-glutamate.

**Data collection and analysis.** In the present study, we analyzed the innervation of identified tracheal vagal preganglionic neurons by TH-containing fibers. All labeled axodendritic and axosomatic synapses that were observed on retrogradely labeled tracheal AVPNs within the rNA were counted. In another series of experiments, two to three sections from the rNA region of each animal were used to examine the expression of α2A-ARs on tracheal AVPNs. Immunostained tissue sections were examined by using an Olympus AX70 fluorescence microscope (Olympus America, New York, NY). For each trait, the intensity of the signal for immunolabeled neurons and the intensity of the background signal were measured by using Sigma Scan Pro image anal-
analysis software (SPSS, Chicago, IL). In addition, a Leica TCS-SP2 laser scanning confocal microscope was used to examine the TR and FITC fluorescence, because this technique has higher sensitivity and specificity in detecting two fluorescent labels in a single neuron within relatively thick sections. With both methods, α2A-AR immunoreactivity and CTb-immunoreactive sites in the rNA were confined to the cytoplasm and dendrites. Digital images of the CTb-specific and α2A-AR-specific staining were obtained from the same part of the tissue section in regions of the rNA that contained identified AVPNs. Only neurons in which the CTb staining outlined the entire cell body and had an α2A-AR-specific immunoreactivity at least threefold above background were considered to manifest robust expression of the α2A-AR. Norepinephrine concentrations were expressed in femtograms per microliter. Records from physiological experiments were analyzed to determine the airway responses to LC stimulation before and after interventions. Average values of each variable are presented as means ± SE. Statistical comparisons were made by using the Student’s t-test or a two-way analysis of variance when appropriate. The criterion for statistical significance was P < 0.05.

RESULTS

Ultrastructural studies. In previous studies in ferrets (22), retrogradely labeled AVPNs were observed in two regions: the rostral part of the dorsal motor nucleus of the vagus and the rostral nucleus ambiguus. We focused on the retrogradely labeled vagal preganglionic neurons innervating the extrathoracic trachea within the nucleus ambiguus, because these neurons provide the major cholinergic outflow to the airways. These cells were readily detectable in the electron microscope, owing to the presence of an electron-dense crystalline TMB-tungstate reaction product, primarily in the cytoplasm of the cell (Fig. 2) and proximal dendrites. However, a few labeled distal dendrites were also detected. The axons of TH neurons within the rNA were found to be unmyelinated and intermingled with other myelinated and unmyelinated axons that were not immunoreactive for TH (Fig. 3A). TH-immunoreactive terminals contained a mixed population of small, clear, pleomorphic vesicles as well as several large, dense core vesicles. These terminals formed synapses with unlabeled profiles in the NA (Fig. 3C); however, only 1 of 512 synapses observed on the perikarya or dendrites of AVPNs was immunoreactive for TR (Fig. 3D).

α2A-AR immunocytochemistry studies. To demonstrate whether α2A-ARs were expressed by identified vagal preganglionic neurons innervating the trachea, double-labeling immunocytochemistry studies were performed. Fluorescence microscopic analysis of brain stem sections obtained from six ferrets demonstrated that AVPNs express different levels of α2A-AR. Our analysis indicated that 372 out of 747 CTb-positive cells (49.8%) manifested robust α2A-AR-like immunoreactivity. There were also profiles of robust α2A-AR-specific staining in neurons in which no CTb staining was detected. We counted 1,088 α2A-AR-immunoreactive CTb unlabeled neurons within the rNA and the surrounding region. Using a Leica TCS-SP2 laser scanning confocal microscope, we examined codistribution of α2A-AR-specific staining in AVPNs retrogradely labeled after CTb injection into the wall of the extrathoracic trachea. Many retrogradely labeled AVPNs (Fig. 4A), were observed to express α2A-AR-specific staining (Fig. 4B). α2A-AR-specific staining was also observed on dendrites of the AVPNs. This is clearly observable in the overlay images (Fig. 4, C and D), characterized by the yellowish perikarya due to the overlap of CTb-specific (FITC, green) and α2A-AR-specific (TR, red) staining. In control experiments, there was no apparent cross-reactivity of the secondary antibodies (data not shown).

Microdialysis and HPLC measurements of norepinephrine release. Norepinephrine release within the rNA region before and after LC stimulation was studied in eight ferrets. Three weeks before microdialysis experiments, four of these animals were ovalbumin sensitized. Repeated stimulation of LC neurons with glutamate microinjections at 5-min intervals elicited an increase in norepinephrine levels within the AVPN region. No differences were found in a control state and after LC stimulation-induced release of norepinephrine between sensitized and nonsensitized ferrets. Hence the data were combined. Typical HPLC chromatograms of microdialysates collected from the rNA in a control state and after repeated LC stimulation are presented in Fig. 5. After equilibration of the dialysis probe, but before stimulation of the LC region, the basal levels of norepinephrine were determined by using the first three fractions collected before the intervention in eight animals. The extracellular norepinephrine levels were 28.2 ± 4.5 fg/μl. After LC stimulation, the extracellular concentration of norepinephrine increased. As seen in Fig. 5, the peaks of norepinephrine release were slightly higher in the second fraction, occurring 20 min after the last LC stimulation. The average peak concentration of norepinephrine, measured as a higher concentration in the first or in the second fraction, was 80.6 ± 13.7 fg/μl, significantly different from baseline.

**Fig. 2.** Example of electron microscope image of a labeled tracheal AVPN (N) readily identifiable by the presence of crystalline tetramethylbenzidine tungstate reaction product (large arrow) in the cytoplasm of the cell. An unlabeled neuron (n) is identified for comparison. Bar = 2 μm.
concentrations \((P < 0.05)\). Norepinephrine levels returned to normal within 40–60 min after the LC stimulation.

In three control ferrets, in which no LC stimulation was performed, extracellular concentrations of measured norepinephrine in samples collected at the same time points as in experiments with LC stimulation were stable (Fig. 5), indicating that any change in extracellular norepinephrine was due to the stimulation of LC norepinephrine-containing cells.

**Physiological experiments.** In eight healthy and non-sensitized ferrets, before \(\alpha_{2A}\)-adrenergic receptor blockade, activation of LC neurons by microinjection of glutamate caused a decrease in tracheal tone, which was not affected by prior blockade of \(\beta\)-adrenergic receptors. Tracheal pressure started to decline within 3–5 s; maximal depressive effects were noted within 2 min after LC stimulation and gradually returned to prestimulation values. After full recovery, repeated LC stimulation caused comparable decreases in tracheal tone. In general, changes in tracheal tone were associated with no statistically significant change in either arterial pressure or heart rate (mean arterial pressure before stimulation \(143 \pm 6\) vs. \(136 \pm 4\) mmHg after stimulation \((P > 0.05)\) and heart rate \(355 \pm 11\) vs. \(344 \pm 12\) beats/min \((P > 0.05)\).

An example of the airway smooth muscle tone response to unilateral injection of 4 nmol of glutamate into the LC region is presented in Fig. 6A. In this animal, before blockade of \(\alpha_{2A}\)-AR, LC stimulation caused tracheal pressure to decrease from 32 to 14 cmH\(_2\)O. Blockade of \(\alpha_{2A}\)-AR by bilateral microperfusion of yohimbine into the rNA diminished the decrease in tracheal smooth muscle tone elicited by activation of LC neurons. Furthermore, tracheal pressure returned to baseline levels faster than before \(\alpha_{2A}\)-AR blockade. In control periods after LC stimulation, Ptseg decreased on average by 16.7 cmH\(_2\)O. After \(\alpha_{2A}\)-AR blockade, in response to LC stimulation, tracheal pressure declined on average only by 5.5 cmH\(_2\)O. The
difference of tracheal tone response to LC stimulation before and after blockade of α2A-AR was significant (P < 0.05). After blockade of α2A-AR, LC stimulation still had no significant effect on mean arterial pressure or heart rate (data not shown).

DISCUSSION

Ultrastructural studies. The results of the present electron microscopic study showed that, in the ferret, catecholaminergic terminals or varicosities, labeled by TH immunohistochemistry, are found within the rNA. Previous studies (17, 21, 49) have revealed that the majority of catecholaminergic neurons innervating AVPNs were observed in two distinct groups: along the ventrolateral margin of the pontine tegmentum (A5 cell group), and dorsal and lateral to the midline and beneath the fourth ventricle (locus coeruleus and subcoeruleus). Although the A5 cell group projects to the medulla oblongata and spinal cord, the locus coeruleus and subcoeruleus (A6) have extensive ascending and descending projections throughout the neuraxis (46, 55). The contribution of catecholaminergic neurons lo-

![Image](http://example.com/image1.png)

Fig. 4. Example of a confocal microscope image of α2A-adrenergic receptor subunit (α2A-AR) expression by the AVPNs innervating the extrathoracic trachea. A: CTb-labeled neurons were identified by using a fluorescein-conjugated antibody (FITC, green). B: specific α2A-AR staining is observed on the membrane of the perikaryon as well as on dendrites of neurons within the rNA, visualized by a Texas Red-conjugated secondary antibody (TR, red). C: overlay (superimposed) images in which the overlap of CTb-specific (FITC, green) and α2A-AR-specific (TR, red) signals generate a yellowish color. D: higher power image of the area indicated by the box in C. In control experiments, there was no apparent cross-reactivity of the secondary antibodies. Bar = 40 μm (A–C); 20 μm (D).

![Image](http://example.com/image2.png)

Fig. 5. A: typical HPLC chromatograms obtained from microdialysates collected from airway-related vagal preganglionic motor neurons within the rNA in a control state (Baseline) and during repeated excitation of LC neurons (Stimulation). B: average results (mean ± SE; n = 8) of norepinephrine in the control state and at different time points after cessation of chemical stimulation (horizontal bar). In 3 control animals, no stimulation was performed. NE, norepinephrine. *P < 0.05.
cated in the ventrolateral medullary reticular formation (A1 and C1 cell groups) and in the dorsal aspect of the medulla oblongata (A2 and C2 cell groups) to noradrenergic or adrenergic innervation of AVPNs was found to be relatively small (21).

The effects of endogenously released norepinephrine can be conveyed through specialized membrane junctions, i.e., via synaptic transmission. In addition, the transfer of information may occur nonsynthetically, using the extracellular space as a communication channel, i.e., volume transmission (1). Neuroanatomical results of the present study suggest that the modulatory effects of norepinephrine on cholinergic outflow to the airways are mainly exerted by nonsynaptic actions, because only a few unequivocally identifiable TH-containing synapses were observed on the identified vagal preganglionic motor neurons innervating the trachea. Because the origin of the noradrenergic nerve terminals in the cerebral cortex is primarily from the LC, it is of interest to note that a previous study concluded that the cortical noradrenergic innervation is also mediated primarily by volume transmission (7). On the other hand, data from this laboratory have shown that TH-immunoreactive nerve terminals in more caudal regions of the nucleus ambiguous form distinct synapses on the negative inotropic vagal preganglionic neurons projecting to the heart (39).

The quantitative analysis of brain tissues processed for electron microscopic immunocytochemistry is relatively imprecise. This is because the quality of immunocytochemical labeling is usually inversely proportional to the degree of ultrastructural tissue preservation. Thus, as ultrastructure is preserved with strong fixatives, the immunocytochemical labeling that can be demonstrated is often severely degraded. How confident, therefore, can we be that there are, in fact, few direct synapses of TH terminals on AVPNs in the rNA? In this regard, we would note that it is highly unlikely that inefficient antibody penetration contributed to an underestimation, because our tissues were pretreated with ethanol to facilitate antibody penetration. Furthermore, all tissue sections to be analyzed for electron microscopy were cut in their entirety with an ultramicrotome, and robust TH labeling was observed throughout the sections. Because only 0.2% of the terminals that formed synapses with tracheal AVPNs in the rNA were immunoreactive for TH, it is probable that the released norepinephrine reaches extrasynaptic membrane receptors on AVPNs by diffusion, volume transmission, and to a substantially lesser extent via direct synaptic communication.

**α₂AR immunocytochemistry studies.** The results of the present study show that α₂ARs are densely present on the somata and dendrites of AVPNs, participating, as a heteroreceptor, in the regulation of cholinergic outflow to the airways and airway smooth muscle tone. This agrees with previous studies in other species showing that α₂AR-immunoreactive cells are not limited to catecholaminergic cells but is present also in other cells, including the large glutamatergic neurons of the lateral reticular nucleus (5, 16), however, not in GABAergic cells (44).

It has been shown that α₂-ARs are expressed on nerve terminals, where their activation could inhibit neurotransmitter release (4, 14). Hence, α₂-AR antagonists may produce an opposite action from an agonist, suggesting that α₂-ARs may be tonically active. Conceivably, the experimental design of the present experiments did not allow us to observe the expression of α₂-ARs on excitatory nerve terminals innervating AVPNs and their tonic inhibitory influence on cholinergic outflow to the airways; however, future studies will more directly address this issue.

In contrast to AVPNs, several cranial nerve motor nuclei, including those from which the hypoglossal nerve arises, express α₁-adrenergic receptors. Thus the postsynaptic excitatory effects of norepinephrine on hypoglossal motoneurons must be primarily mediated by α₁-adrenoceptors (58). Furthermore, norepinephrine acting via α₁-adrenoceptors, probably through a decrease in postsynaptic leak K⁺ conductance, increases the excitability of both the central respiratory command and spinal inspiratory output cells (47) but inhibits the activity of vagal preganglionic neurons innervating the extrathoracic trachea.

**Microdialysis and HPLC measurements of norepinephrine release.** The results of the present study showed for the first time that stimulation of the LC and...
subcoeruleus region elicited a release of norepinephrine within the rNA, which gradually returned to normal. These results further support the contention that in vivo microdialysis can be used for studying chemical neurotransmission and neurochemical characterization of brain circuitry (56). Although this approach possesses high specificity, it lacks temporal resolution, because of the long sampling times needed to accommodate the low flow rates of perfusate through the probe. Another possible limitation is that the size of the probe reduces the anatomic specificity of the field from which the dialysate is collected. However, the application of microdialysis sampling using probes dimensioned for a rat or a mouse (12, 30, 62), as in the present studies, is more feasible in larger animals, such as a ferret. Furthermore, an ultrasensitive HPLC method for the determination of norepinephrine by electrochemical detection makes it possible to measure basal levels of norepinephrine in the rNA regions in femtogram per microliter concentrations.

In the present study, we observed a delayed return of norepinephrine to prestimulation levels. There is no solid ground for reasonable explanation of this observation. It could be due to repeated and sustained activation of noradrenergic neurons, the slow uptake mechanisms, or both.

**Physiological experiments.** The findings of the present study indicate for the first time that LC stimulation induces centrally mediated inhibition of cholinergic outflow to the airways and a consequent airway smooth muscle relaxation. In the ferret, excitatory innervation of the airways is exclusively cholinergic, and a nonadrenergic, noncholinergic inhibitory system does not appear to play an important role in regulation of tracheal smooth muscle tone (43). This is also true for the cervical trachea of the cat (11); however, in the trachea of the guinea pig (41), or intrapulmonary airways of the cat (9), the opposite is true. There is evidence that vasoactive intestinal peptide is present in airway ganglionic neurons (8), and when released by electrical field stimulation, participates in mediating nonadrenergic, noncholinergic relaxation of guinea pig tracheal strips (3). However, to our knowledge, there are no published data showing that activation of AVPNs induces vasoactive intestinal peptide release within the airways.

In ferrets, an alternative explanation for airway smooth muscle relaxation after stimulation of the LC could be an increase in sympathetic outflow. However, in the present study airway dilation elicited by LC stimulation was resistant to β-adrenergic receptor blockade by propranolol, which antagonizes the effects of sympathetic nerve stimulation (11) but does not affect the release of norepinephrine within the central nervous system induced by chemical activation of LC neurons (29). The observed decrease of airway smooth muscle tone induced by stimulation of the LC neurons might also arise from a modulation of baroreceptor inputs. Changes in arterial pressure result in alterations of airway tone that are in the opposite direction (53), and in the present study activation of LC neurons had no significant effect on arterial blood pressure.

It is possible that centrally released norepinephrine may activate GABAergic interneurons that project to vagal preganglionic cells innervating the airways, as in other brain regions, via activation of α1- and β-ARs (42, 52). Our previous studies showed that AVPNs express GABAα receptors, and GABA inhibits cholinergic outflow to the airways (24). However, the changes that we observed cannot be explained solely by the activation of GABAergic mechanisms, because prior blockade of α2ARs within rNA region significantly reduced the airway smooth muscle relaxation induced by LC stimulation.

**Physiological relevance of noradrenergic innervation of the AVPNs.** The physiological relevance of catecholaminergic innervation of the AVPNs and of the α2 ARs expressed by these cells is not well understood. It is possible that these monoaminergic neurons play an important role in a dynamic gain control of the excitability of vagal preganglionic neurons innervating the airways, regulating their discharge during behavioral and emotional changes, such as exercise and stress. Exercise increases the levels of norepinephrine in the brain regions innervated by the LC (30) and in humans induces airway dilation due to inhibition of resting vagal tone (59), through the muscle reflex (31). Alterations in noradrenergic control of AVPNs may contribute to exercise-induced asthma.

The firing rate of noradrenergic neurons progressively changes across the sleep-wake-arousal cycle: diminishes in quiet sleep, is abolished when entering active rapid-eye-movement sleep, and is dramatically elevated during arousal (2, 57). Therefore, reciprocal changes in airway smooth muscle tone may occur with fluctuations in the activity of norepinephrine-containing cells that project to the AVPNs. This hypothesis is supported by findings showing that, in humans, airway caliber also undergoes cyclic oscillations: decreases at night and increases during the day. The fluctuations are greatly amplified in patients with nocturnal worsening of asthma (34, 38, 54) and might be partly due to withdrawal of the inhibitory influences that in turn will trigger the cascade of events that enhances airway narrowing and nocturnal worsening of airway conductivity.

Neural mechanisms play an important role in airway hyperreactivity (26). Fear and emotional distress may facilitate the occurrence of bronchoconstrictive attacks (33). Previous receptor binding studies have shown that chronic psychosocial stress downregulates the binding sites for α2-adrenergic ligands in several brain sites (13, 44). Although the receptor changes on AVPNs have not yet been analyzed, the stress-induced downregulation of receptor expression on AVPNs may contribute to an imbalance in the excitatory and inhibitory inputs to AVPNs. In addition, chronic stress reduces expression of α2-adrenoceptor in glutamatergic neurons of the brain stem (44). Because glutamate is the main excitatory neurotransmitter involved in reflex bronchoconstriction (19, 23), the stress-induced down-
regulation in α2-adrenoceptor expression in these neurons might augment airway bronchoconstrictive reflex responses, via increased glutamatergic drive to the vagal preganglionic neurons innervating the tracheobronchial system.

In conclusion, the results of the present study strongly support the concept that central noradrenergic inhibitory pathways participate in the regulation of the cholinergic drive to the tracheobronchial system, mainly via volume (nonsynaptic) transmission and to a lesser extent through wiring (synaptic) connectivity. Downregulation of these influences may result in a shift from inhibitory to excitatory influences, leading to a hyperexcitable state of the AVPNs and to airway hyperreactivity. Hence, drugs that potentiate central noradrenergic mechanisms, via the activation of α2-adrenoceptors expressed by airway-related vagal preganglionic premotor neurons, may represent targets for the development of new treatments for airway overreactivity and increased cholinergic outflow to the airways.

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