Modulation of ACh release from airway cholinergic nerves in horses with recurrent airway obstruction

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Zhang, Xiang-Yang, N. Edward Robinson, and Feng-Xia Zhu. Modulation of ACh release from airway cholinergic nerves in horses with recurrent airway obstruction. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L769–L775, 1999.—To evaluate the functional status of neuronal α2-adrenoceptors (ARs) and β2-ARs on ACh release in horses with recurrent airway obstruction (RAO), we examined the effects of the physiological agonists epinephrine (Epi) and norepinephrine (NE) and the β2-agonists RR- and RR/SS-formoterol on ACh release from airway cholinergic nerves of horses with RAO. Because SS-formoterol, a distomer of the β2-agonist, increases ACh release from airways of control horses only after the autoinhibitory muscarinic receptors are blocked by atropine, we also tested the hypothesis that if there is an M2-receptor dysfunction in equine RAO, SS-formoterol should increase ACh release even in the absence of atropine. ACh release was evoked by electrical field stimulation and measured by HPLC. Epi and NE caused less inhibition of ACh release in horses with RAO than in control horses. At the catecholamine concentration achieved during exercise (10−7 M), the inhibition induced by Epi and NE was 10.8 ± 13.2 and 3.4 ± 6.8%, respectively, in equine RAO versus 41.0 ± 6.4 and 27.1 ± 5.6%, respectively, in control horses. RR- and RR/SS-formoterol (10−8 to 10−5 M) increased ACh release to a similar magnitude as that in control horses. These results indicate that neuronal β2-ARs are functioning; however, the α2-ARs are dysfunctional in the airways of horses with RAO in response to circulating catecholamines. SS-formoterol (10−8 to 10−5 M) facilitated ACh release in horses with RAO even in the absence of atropine. Addition of atropine did not cause significantly more augmentation of ACh release over the effect of SS-formoterol alone. The magnitude of augmentation in horses with RAO in the absence of atropine was similar to that in control horses in the presence of atropine. The latter observations could be explained by neuronal muscarinic-autoreceptor dysfunction in equine RAO.

adrenoceptor; prejunctional; muscarinic receptor; catecholamine; formoterol; enantiomer; autoreceptor

Acute exacerbations of recurrent airway obstruction (RAO) of horses are characterized by bronchospasm, inflammation of the tracheobronchial tree, and nonspecific airway hyperresponsiveness (14). The rapid decrease in pulmonary resistance and increase in dynamic compliance after muscarinic blockade with atropine indicates that a large part of the bronchospasm is mediated through cholinergic mechanisms (3). The release of ACh from equine airway cholinergic nerves is inhibited prejunctionally by an inhibitory α2-adrenoceptor (AR) (20, 22) and an autoinhibitory muscarinic receptor (18). Dysfunction of these receptors may result in an exaggerated release of ACh, an airway spasmogen, and contribute to the tracheobronchial constriction and hyperresponsiveness in horses with RAO. In normal horses, the α2-AR is activated by levels of circulating catecholamines achieved during exercise (16, 22). Wang et al. (19) have previously demonstrated that the inhibitory effect of the α2-AR agonist clonidine on ACh release is lacking in bronchi and less potent in the trachea of horses with RAO. We wanted to know whether the α2-AR dysfunction is reflected in the response to endogenous catecholamines. Therefore, in the first protocol of the present study, we examined the effect of epinephrine (Epi; 10−8 to 10−5 M) and norepinephrine (NE; 10−8 to 10−5 M) on ACh release from airway parasympathetic nerves of horses with RAO.

Zhang and colleagues (21, 22, 24, 25) were the first to report an excitatory β2-AR in airway parasympathetic nerves. After their report in the horse, this receptor was subsequently reported in guinea pig (2) and human airway parasympathetic nerves (5). In airway preparations of control horses, activation of β2-ARs by isoproterenol, by the racemic mixture of specific β2-agonists such as albuterol and formoterol, or by R-enantiomers of β2-agonists can increase ACh release in a concentration-dependent manner. The maximal augmentation resulting from activation of β2-ARs is approximately twofold (21, 22, 24, 25). To evaluate the neuronal β2-AR function in horses with RAO, in the second protocol of our present study, we examined the effect of the racemic mixture of formoterol (RR/SS-enantiomer) and RR-formoterol on ACh release from tracheal parasympathetic nerves of horses with RAO.

The prejunctional muscarinic autoreceptor provides negative feedback on airway cholinergic nerves, and these receptors are dysfunctional in guinea pigs challenged by ovalbumin (9) or infected with parainfluenza virus (8) and in human asthmatic patients (13). However, the functional status of neuronal M2 receptors in equine RAO is still unknown. SS-formoterol, a distomer of the β2-AR agonist, increases ACh release in the control horses only in the presence of muscarinic blockade with atropine, i.e., when neuronal M2 receptors are dysfunctional (24). Therefore, in the third protocol of the present study, we tested the hypothesis that if there is a prejunctional M2-receptor dysfunction in horses with RAO, SS-formoterol should increase ACh release even in the absence of muscarinic blockade with atropine.
MATERIALS AND METHODS

Animals. The study was approved by the All-University Committee on Animal Use and Care of Michigan State University (East Lansing). Six horses (body wt 481.7 ± 29.0 kg, age 14.5 ± 1.2 yr) with RAO during acute exacerbation were studied. Several days before the experiment, the RAO-susceptible horses were transferred from the pasture to stalls where they were fed dusty hay (6). They remained in the stalls until clinical signs of airflow obstruction, such as flared nostrils and forced abdominal effort during expiration, were observed.

Tissue preparation. The animals were euthanized by injection of an overdose of pentobarbital sodium through the jugular vein. Other investigators also used tissues from the same animals for a variety of studies. Postmortem examination revealed that the lungs remained inflated when the chest was opened, and plugs of mucus and exudate were observed in the airways of all the animals. A segment of the trachea between the 16th and 30th cartilaginous rings above the carina was quickly collected, immersed in Krebs-Henseleit (KH) solution (composition in mM: 118.4 NaCl, 25.0 NaHCO3, 11.7 dextrose, 4.7 KCl, 2.6 CaCl2·2H2O, 1.19 MgSO4·7H2O, and 1.16 KH2PO4), and gassed with 95% O2-5% CO2 during the whole experiment. The trachea was opened longitudinally in its anterior aspect by dissection of the cartilages and was pegged flat on a paraffin block submerged in KH solution. Tracheal smooth muscle strips with the epithelium intact were cut with a template along the fiber direction before being suspended in tissue baths. The temperature within the baths was maintained at 37°C, and the KH solution was changed every 15 min. Square-wave electrical impulses were produced by a stimulator (S88, Grass Instruments, Quincy, MA) and passed through a stimulus power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO) to electrodes in the tissue baths.

Measurement of electrical field stimulation-induced ACh release. Four tissue strips (each measuring 2 × 15 mm) were cut and tied together at both their ends with 3-0 surgical silk thread. Each trachealis strip bundle (wet weight 217.3 ± 3.3 mg; n = 72 strips) was suspended in a 2-ml tissue bath with a pair of parallel platinum wire electrodes built against the wall of the bath in the vertical direction (Radnoti Glass Technology, Monrovia, CA). One end of the tissue strip was secured to the bottom of the bath with a glass tissue holder; the other end was attached to an 8-g weight via a surgical thread that passed over a steel bar above the tissue bath (17). After an ~120-min equilibration period, the tissues were incubated for 60 min with the cholinesterase inhibitor neostigmine (10⁻⁶ M) and the sympathetic nerve blocker guanethidine (10⁻⁵ M) with and without the muscarinic-autoreceptor antagonist atropine (10⁻⁷ M). These agents were present during the remainder of the experiment.

The ACh concentration in the tissue bath liquid was measured by HPLC coupled with electrochemical detection. The mobile phase contained 100 mM Na2HPO4 (pH 8.0), and the flow rate was 0.35 ml/min. The samples were filtered through 0.2-µm nylon membrane filters (Acrodiscs 13, Gelman Sciences, Ann Arbor, MI) and injected into the HPLC column at a volume of 25 µl/injection. An external ACh standard (2.5 pmol in 25 µl) was injected into every six samples, and the concentration of ACh in the samples was calculated based on the bracketed calibration (for details of this technique, see Refs. 17, 22).

Study design. Electrical field stimulation (EFS; 0.5 Hz, 0.5 ms, 20 V) was applied to all the tissues for five 15-min periods, with a 30-min resting interval between consecutive stimuli. During the first EFS, we determined the baseline release of ACh. The tested drugs were then added to the baths 10 min before subsequent EFS. To eliminate any ACh that may have been released during the incubation period (17), the tissue baths were drained and refilled with fresh KH solution containing the tested drugs immediately before EFS was begun. The tissue bath solution was collected on the completion of each EFS for the measurement of ACh. The tissues were rinsed four times with the KH solution immediately after collection of the samples. At the end of the experiment, the tissues were blotted dry and weighed.

Protocol 1: Effects of Epi and NE on EFS-induced ACh release from trachealis cholinergic nerves of horses with RAO. Three tissue-strip bundles from each horse were used in the presence of the muscarinic-autoreceptor blocker atropine. One did not receive tested drug treatment and served as the time control. The second and third tissue-strip bundles each received Epi (10⁻⁸ to 10⁻⁵ M) and NE (10⁻⁵ to 10⁻³ M). The results of the effects of Epi and NE on ACh release obtained from the horses with RAO were compared with those of control horses in a previously published study from our laboratory (22).

Protocol 2: Effects of RR- and RR/SS-formoterol on EFS-induced ACh release from trachealis cholinergic nerves of horses with RAO. Six tissue-strip bundles from each horse were used. Three tissue-strip bundles were used in the absence of the muscarinic-autoreceptor blocker atropine. One did not receive β₂-agonists and served as the time control. The remaining two received either RR- or RR/SS-formoterol (10⁻⁸ to 10⁻⁵ M) after the first EFS. A similar protocol was repeated with the other two tissue-strip bundles in the presence of muscarinic-autoreceptor blockade with atropine (10⁻⁷ M). The magnitude of augmentation of ACh release in horses with RAO was compared with that in control horses (24) to evaluate the neuronal β₂-AR function in horses with RAO.

Protocol 3: Effects of SS-formoterol on EFS-induced ACh release from trachealis cholinergic nerves of horses with RAO. Four tissue-strip bundles from each horse were used. Two tissue-strip bundles were used in the absence of the muscarinic-autoreceptor blocker atropine. One did not receive SS-formoterol and served as the time control. The other received SS-formoterol (10⁻⁵ to 10⁻³ M). A similar protocol was repeated with the other two tissue-strip bundles in the presence of muscarinic-autoreceptor blockade with atropine (10⁻⁷ M). The results of the effects of SS-formoterol on ACh release obtained from the horses with RAO were compared with those of control horses (24) to evaluate the neuronal muscarinic function in horses with RAO.

Drugs. Atropine sulfate, acetylcholine chloride, neostigmine methylsulfate, guanethidine monosulfate (all from Sigma, St. Louis, MO), and enantiomers of formoterol (Sepracor, Marlborough, MA) were dissolved and diluted in KH solution. Epinephrine bitartrate and norepinephrine hydrochloride (Sigma) were dissolved and diluted in a 0.1% ascorbate solution. All the drugs were prepared on the day of the experiment. The drug solution was pipetted into the tissue bath at 1% of the bath volume. The final concentration of the drugs was expressed as their bath molar concentration.

Data analysis. The results of the present study obtained from horses with RAO were compared with data from historical control horses studied by the same personnel in the same laboratory. The ACh release rate is expressed in both pico-moles per gram per minute and a percentage of baseline (first EFS without drug treatment). Means ± SE for all parameters were calculated; n is the number of horses studied except where otherwise specified. The percent inhibition of Epi and NE at each concentration (10⁻⁸ to 10⁻⁵ M) on ACh release in horses with RAO was compared with that in control horses (22) with unpaired Student’s t-test. The other data were evaluated by repeated-measures ANOVA and ANOVA with
contrasts with Statview II (Abacus Concepts, Calabasas, CA) for the Macintosh computer. Means were compared by Fisher’s (protected) least significant difference test. P < 0.05 was considered significant.

RESULTS

In the absence of atropine, EFS-induced baseline ACh release averaged 1.55 ± 0.11 pmol·g⁻¹·min⁻¹ (n = 24 strips). This value was not significantly different from that in the control horses (1.39 ± 0.08 pmol·g⁻¹·min⁻¹; Ref. 24). In the presence of blockade of muscarinic receptors with atropine (10⁻⁷ M), EFS-induced baseline ACh release reached 5.23 ± 0.27 pmol·g⁻¹·min⁻¹ (n = 48 strips). This rate of ACh release is similar to that in the control horses (5.50 ± 0.35 pmol·g⁻¹·min⁻¹; Ref. 24).

Protocol 1: Effects of Epi and NE on EFS-induced ACh release from trachealis cholinergic nerves of horses with RAO. In time-control tissues of horses with RAO, ACh release remained constant throughout the five stimulations. Although the maximal inhibition was similar to that in the control horses (22), the response curve to both Epi and NE was shifted to the right in RAO-affected animals. Epi and NE caused a significant inhibition of ACh release only at the concentrations of 10⁻⁷ and 10⁻⁵ M but not at 10⁻⁷ M or lower (Fig. 1). Figure 2 compares the percent inhibition of ACh release with Epi and NE in airways of control horses (22) and horses with RAO. At the catecholamine concentrations achieved during exercise (10⁻⁷ M), the inhibition induced by Epi and NE was 10.8 ± 13.2 and 3.4 ± 6.8%, respectively, in RAO-affected horses (n = 6). However, in the control horses, the inhibition was 41.0 ± 6.4 and 27.1 ± 5.6%, respectively (22). The differences were significant (Fig. 2).

Protocol 2: Effects of RR- and RR/SS-formoterol on EFS-induced ACh release from trachealis cholinergic nerves of horses with RAO. In the absence of atropine, ACh release from time-control tissues remained constant throughout the five stimulations. RR- and RR/SS-formoterol augmented ACh release in a concentration-dependent manner (Fig. 3A). The augmentation reached significance at the concentration of 10⁻⁶ M for both RR- and RR/SS-formoterol. At the concentration of 10⁻⁴ M, RR- and RR/SS-formoterol increased ACh release to 199.3 ± 24.6 and 187.5 ± 8.8%, respectively, of baseline (n = 6 animals). This magnitude is similar to that in the control horses (181.9 ± 9.1 and 187.7 ± 22.7%, respectively, of baseline; Ref. 24). In the presence of atropine, RR- and RR/SS-formoterol also increased ACh release in a concentration-dependent manner (Fig. 3B). The maximal augmentation, i.e., an approximate doubling of ACh release that was reached at a concentration of 10⁻⁷ M for both RR- and RR/SS-formoterol, was of similar magnitude to that in the control horses (24).

Protocol 3: Effects of SS-formoterol on EFS-induced ACh release from trachealis cholinergic nerves of horses with RAO. In the control horses, SS-formoterol (10⁻⁸ to 10⁻⁵ M) had no effect on ACh release in the absence of atropine. However, SS-formoterol dose dependently facilitated ACh release in the presence of atropine, and at the concentration of 10⁻⁵ M, SS-formoterol increased the release to 166.1 ± 7.7% of baseline (Fig. 4A; Ref. 24). In the horses with RAO, SS-formoterol (10⁻⁸ to

![Graph 1](http://ajplung.physiology.org/)

Fig. 1. Effect of epinephrine and norepinephrine at indicated concentrations on ACh release in response to electrical field stimulation (20 V, 0.5 ms, 0.5 Hz) of trachealis strips from horses with recurrent airway obstruction (RAO; n = 6). *Significantly different from time control.

![Graph 2](http://ajplung.physiology.org/)

Fig. 2. Comparison of percent inhibition of ACh release with epinephrine (A) and norepinephrine (B) at indicated concentrations in response to electrical field stimulation (20 V, 0.5 ms, 0.5 Hz) of trachealis strips from control horses (n = 6; Ref. 22) and horses with RAO (n = 6). *Significantly different from control horses.
10⁻⁵ M) increased ACh release even in the absence of atropine (Fig. 4B). At the concentration of 10⁻⁵ M, SS-formoterol increased ACh release to 172.3 ± 13.5% of baseline (n = 6 animals). The magnitude of augmentation in the absence of atropine in horses with RAO (Fig. 4B) was similar to that in the control horses in the presence of atropine (Fig. 4A; Ref. 24). The addition of atropine slightly but not significantly increased ACh release over the effect of SS-formoterol alone (Fig. 4B).

**DISCUSSION**

The effect of catecholamines on ACh release from equine airway parasympathetic nerves is mediated by both α₂-inhibitory and β₂-excitatory adrenoceptors, with the former being predominant (22). The sympathetic discharge of the horse increases the mean plasma concentrations of Epi and NE from 9 ± 10⁻¹⁰ and 7 ± 10⁻ⁱ⁰ M, respectively, at rest, to 1.53 ± 10⁻⁷ and 1.48 ± 10⁻⁷ M, respectively, during exercise (16). In a previous study, Zhang et al. (22) demonstrated that both Epi and NE at concentrations of 10⁻⁸ to 10⁻⁵ M dose depen-
creased facilitatory effect of $\beta_2$-AR, or a combination of both. However, our results demonstrated that in airway parasympathetic nerves of the RAO-affected horse, activation of $\beta_2$-ARs with both RR- and RR/SS-formoterol (10$^{-8}$ to 10$^{-3}$ M) increased ACh release with a similar magnitude as that in control horses (24). This result indicated the absence of prejunctional $\beta_2$-AR dysfunction in airway parasympathetic nerves of horses with RAO. Therefore, attenuated inhibition of Epi on ACh release from RAO-affected animals must be due to the dysfunction of prejunctional inhibitory $\alpha_2$-ARs. Because the inhibitory effect of NE is solely mediated via $\alpha_2$-ARs (22), decreased NE-induced inhibition of ACh release from RAO-affected animals provides additional evidence of prejunctional $\alpha_2$-AR dysfunction. Combining all the above evidence, we conclude that the prejunctional $\alpha_2$-AR is dysfunctional in horses with RAO and that this $\alpha_2$-AR dysfunction is reflected in the response to endogenous catecholamines (16). However, the function of prejunctional $\beta_2$-ARs is normal.

In the control horses, Zhang et al. (24) have previously demonstrated that SS-formoterol, a distomer of the $\beta_2$-agonist, facilitates ACh release in the presence of atropine, which blocks the prejunctional muscarinic autoinhibitory receptor, but not in the absence of atropine when the neuronal muscarinic autoinhibitory receptor is functional (Fig. 4A). Therefore, we hypothesized that if the prejunctional $M_2$ receptors are dysfunctional in equine RAO, SS-formoterol should increase ACh release even in the absence of muscarinic blockade with atropine. In support of this hypothesis, our results revealed that SS-formoterol facilitated ACh release in the absence of atropine and that the magnitude of augmentation in the horses with RAO in the absence of atropine was similar to that in the control horses in the presence of atropine. Furthermore, in the RAO-affected horses, blockade of muscarinic receptors by atropine did not cause significantly more augmentation of ACh release over the effect of SS-formoterol alone (Fig. 4B). These observations suggest that the prejunctional muscarinic autoreceptors in airway parasympathetic nerves of horses with RAO are dysfunctional.

The neuronal autoinhibitory $M_2$ receptors are also dysfunctional in ovalbumin-sensitized guinea pig airway parasympathetic nerves after antigen challenge (9), in virus-infected guinea pigs (8), and in ozone-challenged guinea pigs (15) and also may be dysfunctional in some asthmatic patients (13). However, the pathogenesis of $M_2$-receptor dysfunction is different in the different diseases. In antigen-challenged guinea pigs (12) and human asthmatic patients (4), there is a large influx of eosinophils in the airway. These eosinophils and their major basic protein, an antagonist for $M_2$ receptors (12), are especially associated with airway nerves (4). In virus-infected guinea pigs, viruses have both an indirect leukocyte-dependent effect and a leukocyte-independent effect on $M_2$ receptors (10). In ozone-challenged guinea pigs, leukocytes or mediators are important in the pathogenesis of the loss of $M_2$-receptor function (11). Equine RAO is a delayed hypersensitivity response to inhaled antigen, particularly the thermophilic molds and actinomycetes that grow in damp hay (14), and is characterized by a predominant neutrophilic inflammation with few eosinophils as revealed by bronchoalveolar lavage (7). However, the role of inflammation or mediators in the development of neuronal $M_2$-receptor dysfunction in equine RAO is unknown, and further investigations are needed.

Despite the apparent $M_2$-receptor dysfunction, we noticed that the magnitude of RR- and RR/SS-formoterol-induced augmentation of ACh release was similar in the two groups of animals and that the amount of ACh release evoked by EFS in picomoles per gram per minute in equine RAO was not different from that in the control horses. Why should the neuronal $M_2$-receptor dysfunction be reflected only in the response to SS-formoterol, a weak $\beta_2$-agonist, but not in the response to the potent $\beta_2$-agonists (RR- and RR/SS-formoterol) or EFS-induced baseline ACh release? Previous studies in airway parasympathetic nerves (23) and the myenteric plexus (1) suggest that neuronal $M_2$ receptor-mediated inhibition of ACh release is via at least two different intracellular mechanisms: cAMP-dependent and cAMP-independent pathways, with the latter predominant, whereas the facilitation of ACh release by $\beta_2$-AR agonists involves only cAMP-dependent pathways (23). These two pathways are diagrammed in Fig. 5. If neuronal $M_2$-receptor dysfunction in equine RAO affects the cAMP-dependent but not the cAMP-independent pathway, the dysfunction of the $M_2$-receptor should result in decreased ACh release, but the release should still be augmented with formoterol.
receptors would be reflected primarily in their responses to agents that act via intracellular CAMP. Under this scenario, activation of β2-AR by a weak agonist, SS-formoterol, has no effect in control horses because the ACh released during EFS activates the CAMP-dependent autoinhibitory mechanism and prevents the β2-AR agonist-induced increase in CAMP (Fig. 5). However, when the CAMP-dependent autoinhibitory pathway is dysfunctional, the weak agonist increases CAMP and facilitates release. Such is the situation in RAO-affected horses and in control horse tissue after atropine treatment. By contrast, when the β2-ARs are activated by full β2-AR agonists such as RR- and RR/SS-formoterol, the increase in intracellular CAMP cannot be overridden by the relatively weak CAMP-dependent M2-receptor autoinhibitory pathway and ACh release increases. In this situation, M2-mediated inhibitory CAMP-dependent pathway dysfunction is irrelevant because the autoinhibition of CAMP production is trivial in comparison to the major increase in CAMP engendered by the strong β2-AR agonist.

A similar explanation can be used to explain the absence of a difference in EFS-induced ACh release between tissues from control and RAO-affected horses, the fact that atropine still increases ACh release in such horses, and even the dysfunction of the prejunctional inhibitory α2-AR. If the primary M2-receptor mechanism is CAMP independent and there is dysfunction only in the CAMP-dependent pathway, it will be very difficult to detect differences in release between diseased and control horses, and atropine will still block the activation of the CAMP-dependent M2-receptor autoinhibitory pathway and therefore increase ACh release (Fig. 5). As with the M2 receptor, the prejunctional inhibitory α2-AR acts primarily via CAMP-independent pathways to a small degree via CAMP-dependent pathways (26). Dysfunction solely in the latter pathway may explain the reduced inhibition of ACh release by lower concentrations of Epi and NE in RAO-affected animals.

In conclusion, the present study demonstrated in the airway of RAO-affected horses that 1) the prejunctional inhibitory α2-AR is dysfunctional, which may lead to less inhibition of bronchospasm in exercising horses with RAO; 2) neuronal β2-AR function is normal; and 3) SS-formoterol-mediated augmentation of ACh release in the absence of atropine could be explained by neuronal M2-receptor dysfunction. Therefore, dysfunction of prejunctional receptors is not a generalized effect but may be restricted to specific pathways.

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