Pre- and postjunctional effects of inflammatory mediators in horse airways

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Olszewski, Michal A., Xiang-Yang Zhang, and N. Edward Robinson. Pre- and postjunctional effects of inflammatory mediators in horse airways. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L327–L333, 1999.—In addition to their direct contractile effects, histamine (Hist), serotonin [5-hydroxytryptamine (5-HT)], and leukotriene (LT) D_4, in low concentrations, dramatically augment electrical field stimulation (EFS)-induced smooth muscle contractions in equine airways. To determine the mechanism of their action, we studied, in tracheal strips, the effect of these mediators on both cholinergically induced tension and the release of ACh from cholinergic nerves. All three mediators synergistically augmented the contraction of the trachealis that was due to release of endogenous ACh, i.e., EFS-induced contraction. These same mediators caused only a small but parallel shift of the ACh concentration-response curve. Comparison of the mediator effects on the responses to endogenous and exogenous ACh suggested a prejunctional effect. However, release of ACh was augmented only by Hist and 5-HT but not by LTD_4. Hist-induced contraction of trachealis was abolished by pyrilamine (H_1-receptor antagonist) but not by ranitidine (H_2-receptor antagonist), whereas thioperamide (H_3-receptor antagonist) shifted the Hist response curve to the left. The augmenting effect of Hist on EFS-induced contraction was abolished by pyrilamine and unaffected by ranitidine or thioperamide. We conclude that inflammatory mediators can increase endogenous cholinergic responses of equine airways via both prejunctional and postjunctional mechanisms. LTD_4 acts solely on smooth muscle, whereas 5-HT and Hist additionally act on neuronal receptors to facilitate release of ACh. Excitatory effects of Hist, i.e., direct contractile effect, and augmentation of endogenous cholinergic response are both mediated via H_1 receptors, whereas the inhibitory H_3 receptors partially oppose the direct contractile effect of this mediator.

trachealis; acetylcholine release; airway smooth muscle; histamine; serotonin; leukotriene D_4

DURING ACUTE EXACERBATIONS of equine recurrent airway obstruction (RAO; also known as chronic obstructive pulmonary disease or heaves), airway inflammation, hyperresponsiveness, and cholinergically mediated bronchospasm lead to severe airway obstruction (14). Even though cholinergically mediated bronchospasm has been clearly demonstrated, the mechanism of increased cholinergic airway tone in RAO is unclear (3). Previous studies (10, 19) of airways isolated from horses with RAO have excluded intrinsic hyperresponsiveness to cholinergic stimulation of airway smooth muscle (ASM) as a potential mechanism. There is also a lack of evidence that central vagal reflexes are a significant mechanism of this bronchospasm (6). Recently, Olszewski et al. (13) proposed that the mediators released locally by mast cells located at the neuromuscular junction (5) enhance the endogenous cholinergic response of ASM and contribute to cholinergically mediated bronchospasm. This was based on our observation that selected mediators implicated in the pathogenesis of RAO, such as histamine (Hist), serotonin [5-hydroxytryptamine (5-HT)], and leukotriene (LT) D_4, dramatically augment electrical field stimulation (EFS)-induced cholinergic contractions of equine small airways of control and RAO-affected horses in vitro (13). Lack of a similar effect of these mediators on the ACh concentration response of ASM suggests that they could act prejunctionally to facilitate release of ACh from cholinergic nerve terminals in small airways. The aim of the present study was to determine whether these mediators exert their effects by acting on the receptors located on ASM or on parasympathetic nerves. We compared the results of tension studies with the direct measurements of ACh release. Because the amount of ACh released from small airways is below the level of detection of our measurement system (15), we used isolated equine tracheal strips in which we can measure both release of ACh and tension responses. This approach also allowed us to determine that the potentialization of endogenous cholinergic contractions by inflammatory mediators in equine airways is not restricted to the small airways but also involves other segments of respiratory tract. Additionally, we defined the role of Hist-receptor subtypes with respect to 1) direct contractile effects of Hist and 2) augmentation of cholinergic responses in equine airways.

MATERIALS AND METHODS

Animals and Tissue Collection

For this study, which was approved by the All-University Committee on Animal Use and Care at Michigan State University (East Lansing), we used tissue from 21 clinically healthy horses, geldings, and mares of various breeds 7.1±1.1 yr old and weighing 906.7±25.1 kg. Other investigators also used the tissue from these animals for a variety of studies. For several weeks before entering the study, the animals were carefully monitored to ensure the absence of clinical signs of respiratory disease. After euthanasia with an intravenous injection of pentobarbital sodium, the heart, lung, and trachea were excised from the opened thorax and examined for gross appearance to exclude abnormalities and subclinical lung disease. Immediately, portions of the trachea from 6 to 25 rings above the carina were collected and suspended in Krebs-Henseleit (K-H) solution (composition in mM: 118.4 NaCl, 25.0 NaHCO_3, 11.7 dextrose, 4.7 KCl, 2.6
CaCl₂·2H₂O, 1.19 MgSO₄·7H₂O, and 1.16 KH₂PO₄). During dissection and experimental protocols, the tissues were kept in K-H solution that was continuously gassed with 95% O₂-5% CO₂.

Tissue Preparation
We obtained strips of ASM with intact mucosa by cutting with a template along the direction of the ASM fibers in the longitudinally opened tracheae pinned on a wax plate under K-H solution. Silk ties were placed on the strips 8 mm apart (for tension study) and 15 mm apart (for ACh release) to obtain 8 × 2- and 15 × 2-mm strips, respectively. Individual strips for the tension measurements or bundles of four strips for the ACh measurements were secured at the bottom of 2-ml tissue baths (Radnoti Glass Technology, Monrovia, CA), with the glass tissue holder between pairs of platinum electrodes integrated with the bath walls. The baths were filled with K-H solution maintained at 37°C, and the solution was continuously gassed and replaced every 15 min unless other timing was required by the protocols. Square electric impulses (0.1–16 Hz, 20 V, 0.5 ms) were generated by a stimulator (model S88, Grass Instruments, Quincy, MA) and passed onto the electrodes via a stimulus power booster (Stimu-Splitter II, Med Lab Instrument, Loveland, CO).

Tension Measurement
Tension was measured by a force transducer (model FT03, Grass Instruments) connected to the upper tie of the tracheal strip and installed on a tension manipulator. The isometric force of the tissue preparations was recorded on a polygraph (model 7D or 7E, Grass Instruments). During equilibration (2 h), 16-Hz EFS was applied for 23 min at 7- to 10-min intervals. Passive tension (2–3 g) was adjusted for each strip to produce the maximal response to EFS. After equilibration, the maximal response to 127 mM KCl-substituted K-H solution was recorded, and the tissues were repeatedly washed until the muscle tension returned to baseline.

Measurement of EFS-Induced ACh Release
After a 2-h equilibration period, the tissues were incubated for 60 min with the cholinesterase inhibitor neostigmine (10⁻⁶ M), the sympathetic nerve blocker guanethidine (10⁻⁵ M), and the muscarinic-autoreceptor antagonist atropine (10⁻⁷ M). These agents were present during the remainder of the experiment. For each measurement, a 15-min period of EFS was applied to induce release of ACh from cholinergic nerve terminals. To eliminate any ACh that may have been released during the incubation period (15), before each EFS, the baths were filled with fresh K-H solution containing the tested drugs. Tissue bath solution was collected on the completion of each EFS for the measurement of ACh. The tissues were rinsed four times with the K-H solution immediately after collection of the samples. At the end of the experiment, the tissues were blotted dry and weighed. 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 every six samples, and the concentration of ACh in the samples was calculated based on the bracketed calibration.

Study Design
Protocol 1: Effects of LTD₄ on tension response and release of ACh. A treated strip and a control strip were stimulated simultaneously during the entire protocol. Both tissues were stimulated with EFS (0.1, 0.5, 2, 8, and 16 Hz) to create a primary cumulative frequency-response curve. Subsequently, three frequency-response curves were performed in the presence of increasing concentrations of LTD₄ (10⁻⁹ to 10⁻⁷ M). Between curves, a 30-min resting period was allowed, during which the next concentration of LTD₄ was added 15 min before EFS. After the second EFS curve and a 45-min resting or washout period, the effect of LTD₄ on the tension response to exogenous ACh was determined. The treated bath, but not the control bath, received 10⁻⁷ M LTD₄. After 15 min of incubation, ACh (10⁻⁸ to 10⁻³ M) was added to both tissue baths in logarithmic increments to create cumulative concentration-response curves.

ACh release was measured in a separate set of tissue baths for control and treated-strip bundles. One frequency of EFS (0.5 Hz) was applied to all the tissues in four 15-min periods, with a 30-min resting interval between consecutive stimuli. During the first EFS, baseline release of ACh was determined. The subsequent three stimulations were performed in the presence of increasing concentrations of LTD₄ (10⁻⁹ to 10⁻⁷ M) in the treated bath similar to the EFS-induced tension experiment.

Protocol 2: Effects of 5-HT on tension response and release of ACh. This protocol was identical with the LTD₄ protocol except that 10⁻⁸ to 10⁻⁶ M 5-HT was used during consecutive EFS tension responses and 10⁻⁶ M 5-HT was used during the ACh concentration-response curve, and ACh release was measured in the presence of 10⁻⁷ to 10⁻⁴ M 5-HT.

Protocol 3: Direct contractile effects of Hist. Hist concentration-response curves were performed in four tracheal strips by the addition of increasing concentrations of Hist (10⁻⁸ to 10⁻³ M). Three of these strips were pretreated, each with a different Hist-receptor antagonist [10⁻⁶ M pyrilamine (H₁), 10⁻⁷ M ranitidine (H₂), or 10⁻⁶ M thioperamide (H₃)] that was present throughout the protocol.

Protocol 4: Effects of Hist on tension response and release of ACh. This protocol was performed on five tissue strips from which three were pretreated with either 10⁻⁶ M pyrilamine, 10⁻⁵ M ranitidine, or 10⁻⁶ M thioperamide. A primary EFS curve (0.1, 0.5, 2, 8, and 16 Hz) was created in the presence of the antagonists. Hist (3 × 10⁻⁶ M) was added to four tissue baths; the fifth was the control bath (1 of 2 strips without antagonist). After 15 min of incubation, the tissues were stimulated with EFS, washed thoroughly with K-H solution, and rested for 30 min.

The same tissues were further used to determine the effect of Hist on the exogenous ACh response. After a 45-min rest or washout period, all the tissue baths except for the control bath received 3 × 10⁻⁶ M Hist and were incubated for 15 min. Subsequently, the exogenous ACh (10⁻⁸ to 10⁻⁴ M) was added to all five tissue baths in logarithmic increments to create concentration-response curves.

To investigate the dose-response relationship of Hist on EFS-induced contractions, we used two tracheal strips, control and treated with increasing concentrations of Hist (10⁻⁸ to 10⁻⁴ M). We used a single-frequency 0.1-Hz EFS because Hist has the greatest effect on the tension produced by this frequency. Each time, EFS was applied for 23 min until a plateau of the tension response was reached. After the tension returned to baseline, a higher concentration of Hist was added in semilogarithmic increments, 8 min of incubation was allowed, and 0.1-Hz EFS was repeated.
The protocol to study the effect of Hist on ACh release was identical to that used for LTD4 and 5-HT, with the exception that Hist (10⁻⁷ to 10⁻⁵ M) was added to the treatment bath.

Drugs

On the day of the experiments, acetylcholine hydrochloride, guanethidine monosulfate, histamine hydrochloride, 5-hydroxytryptamine hydrochloride, neostigmine methylsulfate, pyrilamine maleate, and ranitidine hydrochloride (all from Sigma, St. Louis, MO) were dissolved in deionized water to obtain stock solutions (10⁻² to 1 M) as needed. Thiomermaleate (Sigma) was diluted in DMSO to a 10⁻² M concentration and stored in the freezer in small portions. LTD4 (Calbiochem) was diluted in K-H solution to 10 µM and frozen in portions that were diluted in K-H solution shortly before addition to the tissue baths. Stock solutions of pyrilamine, ranitidine, and thiomere were directly mixed into K-H solution to obtain their final concentrations, other compounds were serially diluted in K-H solution, and each concentration was added to the muscle baths in a volume of 1%. The concentrations of all substances are expressed as their final bath concentration.

Data Analysis

Data are expressed as means ± SE, and n is the number of horses used in each protocol. Tension data (measured in grams) were calculated as a percentage of the response to 127 mM KCl-substituted Krebs-Henseleit solution (%KCl), whereas ACh release represents the percentage of the baseline value obtained during the first EFS without drug treatment. To determine the effects of the mediators on tension responses and ACh release as well as the effects of the Hist-receptor antagonists, we applied repeated-measures or mixed-design two-way ANOVA as appropriate. Post hoc Tukey’s test or simple main effects tests were used to compare means between the treatment groups. Statistical analysis was conducted with SPSS for Windows (version 7.0). Means were accepted to be significantly different at P ≤ 0.05.

RESULTS

Effects of LTD4

Although LTD4 (10⁻⁹ to 10⁻⁷ M) had no significant effect on smooth muscle tension, it had a synergistic effect with EFS responses (n = 5; Fig. 1A). The tension induced by EFS (0.52 Hz) was significantly augmented in the presence of 10⁻⁸ and 10⁻⁷ M LTD4. The concentration of LTD4 (10⁻⁷ M) that dramatically augmented EFS-induced tension also increased the tension response to ACh. This was mostly a parallel shift associated with a slight but insignificant increase in baseline tension (n = 5; Fig. 1B). There was no effect of LTD4 (10⁻⁹ to 10⁻⁷ M) on EFS-induced ACh release (n = 5; Fig. 1C).

Effects of 5-HT

5-HT increased the tension of the airways, causing a significant increase in baseline tension beginning with 10⁻⁷ M 5-HT (n = 6; Fig. 2A). The synergistic effect of 5-HT (10⁻⁸ to 10⁻⁶ M) on EFS responses was very obvious (n = 6; Fig. 2A). 5-HT (10⁻⁶ M) also had a significant effect on the ACh dose-response curve, but, as with LTD4, it was predominantly a parallel shift associated with elevated baseline tension (n = 6; Fig. 2B). ACh release was increased by 10⁻⁷ to 10⁻⁵ M 5-HT (n = 4; Fig. 2C). The maximal augmentation was reached at 10⁻⁶ M 5-HT. At this concentration, the release was 162.3 ± 11.2% of baseline.

Effects of Hist

Hist caused a concentration-dependent increase in smooth muscle tension. Up to a 10⁻⁶ M concentration of Hist, 10⁻⁶ M pyrilamine totally abolished the contraction induced by this mediator. Ranitidine had no effect on the concentration response to Hist. Thiopemaleate caused a small but significant increase in the tension induced by Hist (n = 6; Fig. 3).

In the absence of Hist, we detected no effect of Hist-receptor blockade on the tension response to EFS (data not shown). Treatment of the tissues with 3 × 10⁻⁶ M Hist resulted in a dramatic increase in the
tension induced by EFS (n = 7; Fig. 4A). This augmentation of tension was greatest at 0.1 Hz and could not be prevented by ranitidine or thioperamide. Pyrilamine abolished the effect of Hist on the EFS response just as it eliminated the Hist-induced contractions (compare Figs. 3 and 4A).

There was no significant effect of 3 x 10^{-6} M Hist on the response of tracheal strips to exogenous ACh (n = 7; Fig. 4B), and the tissues treated with Hist and its antagonists were not significantly different from those treated with Hist alone.

Hist (10^{-7} to 10^{-5} M) increased the EFS-induced ACh release in a concentration-dependent manner. Significance was reached at a concentration of 10^{-5} M Hist (n = 8; Fig. 4C). At 10^{-5} M Hist, the release was increased to 189.1 ± 30.3% of baseline, yet considerable variability between different individuals was noticed.

Hist augmented the response to 0.1-Hz EFS in a concentration-dependent fashion. EFS-induced tension was 104.8 ± 8.5% of the KCl standard at 10^{-4} M Hist versus only 23.6 ± 9.4% in the control tissues (n = 5; Fig. 5A). This augmentation became apparent beginning with 3 x 10^{-8} M Hist and reached significance at 10^{-6} M Hist. When the value of increased baseline tension caused by the direct contractile effect of Hist was subtracted from the total tension (n = 5; Fig. 5B), the concentration-dependent effect was still very obvious, indicating the presence of more than an additive effect of Hist with EFS response throughout all ranges of concentration (n = 5; Fig. 5B).

**DISCUSSION**

As previously shown in equine small airways (12, 13) and now also in the trachea, we have demonstrated that inflammatory mediators have the potential to augment endogenous cholinergic responses. This effect is therefore not restricted to peripheral airways but represents a universal phenomenon throughout the respiratory system of horses. Moreover, we report that Hist and 5-HT act prejunctionally to increase ACh release from the airway parasympathetic nerves in vitro. In contrast to Hist and 5-HT, LTD_{4} had no effect on ACh release and thus augmented the endogenous cholinergic response exclusively via a postjunctional mechanism. Thus it becomes evident that the synergism between mediators and ASM responses to cholinergic nerve stimulation can be a result of both prejunctional and postjunctional actions of inflammatory mediators.

Our previous study (13) with small airways in vitro has shown that Hist, 5-HT, and LTD_{4} modulate only the endogenous cholinergic response, whereas the response to exogenous methacholine or ACh remains largely unaffected. These data suggested that the mechanism of action of these mediators may be prejunctional via action on excitatory neuronal receptors, facilitating the release of ACh. Because the amount of ACh released from small airways is beyond the level of detection of the HPLC measurement system (13), we decided to use tracheal strips to test our hypothesis.

The first goal of our study was to confirm that the inflammatory mediators, which strongly modulated
responses to EFS in small airways, have similar effects on this response in the trachea. LTD\(_4\), 5-HT, and Hist all had a direct contractile effect on equine tracheal smooth muscle. However, contractions of tracheal muscle induced by LTD\(_4\) were weaker than those of small airways, and, therefore, greater concentrations of LTD\(_4\) were needed to increase baseline tension in the present study than in our earlier study of small airways (13). In contrast to the effects on EFS response, none of the mediators exerted a synergistic effect on the response to exogenous ACh. Treatment with 3 \times 10^{-6} M Hist had virtually no effect on the ACh-response curve (Fig. 4B), whereas the effect of 5-HT and LTD\(_4\) was predominantly due to a parallel shift associated with baseline tension elevation (Figs. 1B and 2B). This strong effect on EFS response and the minimal or no effect on the response to exogenous ACh suggest that the mechanism of mediator-induced augmentation is prejunctional rather than postjunctional.

To determine whether the prejunctional mechanism was at play and whether it was solely responsible for the augmentation of EFS response, we compared the...
results of the tension study with the measurements of EFS-induced ACh release from cholinergic nerve terminals in tracheal strips. Our results confirmed that both bioactive amines (Hist and 5-HT) do indeed facilitate ACh release, and, therefore, augmentation of the EFS response was caused, at least in part, by their effects on receptors on parasympathetic postganglionic neurons. This is an important finding because, based on results of both in vitro tension studies and whole animal studies (7–9, 11), both Hist and 5-HT have long been suggested to affect ACh release in the airways. To our knowledge, we are the first to confirm, based on direct measurement, an increase in ACh release from airway parasympathetic nerves by these mediators. Even though 5-HT clearly augments ACh release, its initial augmenting effect on the EFS response occurs at a 100-fold lower concentration (Fig. 2, A and C). Therefore, it is unlikely that augmentation of the EFS response by 5-HT is exclusively a prejunctional phenomenon. In the case of Hist, however, significant effects on the EFS-response and ACh-release data occur at similar concentrations (10−6 M), indicating that most, if not all, of the effects of Hist are prejunctional. The story with LTD4 is different. Although LTD4 did not facilitate ACh release from EFS-stimulated strips, it had a synergistic effect on the endogenous cholinergic response. Lack of a prejunctional effect contrasts with the results of our tension study (13) as well as with the study by others (1), who used indirect methods, which have suggested that LTD4 increases ACh release in airways. Our direct measurements of ACh release clearly demonstrate that this is not the case (Fig. 1C). Therefore, in the equine trachea and also quite likely in the small airways, the mechanism of synergism between LTD4 and EFS response is strictly postjunctional.

It is not unusual to find much stronger effects of agonists on the EFS than on the exogenous ACh tension response. This has generally been interpreted as a prejunctional effect, but, as we have demonstrated in several studies, this interpretation should not be made without more direct evidence. For example, both prostaglandin E2, and the β2 agonist isoproterenol cause much greater inhibition of the EFS than of the ACh response of equine ASM, which could suggest prejunctional inhibition (17, 18, 20). Still, direct measurements of ACh release clearly indicate that there is no inhibitory prejunctional effect of prostaglandin E2, and isoproterenol has an excitatory effect on ACh release in equine airways (16, 20). The reason for the greater sensitivity of endogenous than exogenous cholinergic response to postjunctional effects of agonists is unknown, but it is clearly present not only with inhibitors of muscle contraction but also with spasmodens such as LTD4 and 5-HT.

With respect to Hist pharmacology in equine airways, we have also made some interesting observations. Both the direct contractile effect of this mediator and the synergism with the EFS response were mediated via the H1 receptor because both effects could be prevented by the H1-receptor antagonist pyrilamine (Figs. 3 and 4A). We also observed that the H2-receptor antagonist ranitidine was completely without effect, and the H3-receptor antagonist increased sensitivity of the tissue to Hist (Fig. 3). Before the advent of specific H3-receptor antagonists, Chand and Eyre (4) hypothesized the existence of an inhibitory H3 receptor in equine airways but attributed most of the inhibitory effects of Hist to the action of the H2 receptor. Our studies have confirmed the presence of the inhibitory H3 receptor, but we found no role for the H2 receptor in equine airways. Summarizing the effects of the Hist-receptor antagonists, we can conclude that 1) the H1 receptor is dominant and responsible for all excitatory effects in the trachea of horses, 2) the H3 receptor has a weak inhibitory effect that is largely masked by the excitatory action of the H1 receptor, and 3) the H2 receptor appeared to have no effect in this tissue.

Summarizing our results, we have observed that selected mediators, typical for anaphylaxis and reported to increase in horses with heaves, significantly augment the endogenous cholinergic responses of equine airways in vitro. The mechanism of these effects can be either pre- or postjunctional and both and may represent the mechanism responsible for cholinergically mediated bronchospasm during the acute exacerbations of equine RAO and other inflammatory or obstructive airway diseases.

We thank Cathy Berney for technical assistance and Victoria Hoelzer-Maddox and MaryEllen Shea for manuscript preparation. This work was supported by an endowment from the Matilda R. Wilson Fund (Detroit, MI) and by Bayer (Leverkusen, Germany). Address for reprint requests and other correspondence: N. E. Robinson, Dept. of Large Animal Clinical Sciences, Veterinary Medical Center, Michigan State Univ., East Lansing, MI 48824-1314 (E-mail: robinson@cvm.msu.edu).

Received 9 July 1998; accepted in final form 13 April 1999.

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