

Epinastine (WAL 801CL) modulates the noncholinergic contraction in guinea-pig airways *in vitro* by a prejunctional 5-HT₁-like receptor

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ABSTRACT: Electrical field stimulation (EFS) of guinea-pig airways, *in vitro*, evokes an excitatory nonadrenergic noncholinergic (eNANC) contraction mediated by release of tachykinins from sensory nerve endings. Epinastine (WAL 801CL) is an antihistaminic drug with binding affinity at certain other receptors, including α -adrenergic receptors and various serotonin (5-HT) receptor subtypes. It is used in asthma treatment; however, its mechanism of action remains to be fully defined.

We have investigated whether epinastine could modulate the eNANC contraction in guinea-pig airways *in vitro*, and have tried to elucidate its receptor mechanism.

Epinastine (0.1–100 μ M) produced a concentration-dependent inhibition of the noncholinergic contraction, with a maximum inhibition of $91 \pm 7\%$ at 100 μ M. Pretreatment of the tissues with combined 5-HT₁/5-HT₂ antagonists, methysergide (1 μ M) or methiothepin (0.1 μ M), significantly attenuated the inhibitory effect of epinastine on the noncholinergic contraction. Pretreatment with tropisetron (1 μ M), a 5-HT₃ antagonist, ketanserin (10 μ M), a 5-HT₂ antagonist, thioperamide (10 μ M), a histamine H₃ antagonist, or phentolamine (10 μ M), an α -adrenergic antagonist, however, had no effect. Chlorpheniramine (10 μ M), another histamine H₁ receptor antagonist without significant 5-HT receptor binding affinity, did not produce any inhibition of the eNANC contraction. Epinastine (100 μ M) did not displace the dose-response curve to exogenously applied substance P (0.01–10 μ M).

These results suggest that epinastine, although identified as a 5-HT antagonist, acts as a 5-HT₁ agonist and that it inhibits the noncholinergic contraction in guinea-pig airways through stimulation of a prejunctional 5-HT₁-like receptor, located to sensory nerves.

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Epinastine (WAL 801 CL; Alesion®, CAS 108929-04-0) is a tetracyclic guanidine that shows structural similarity to mianserin, an antidepressant drug with antihistamine and 5-HT₂ antagonistic properties [1]. Epinastine was originally introduced as an antihistamine drug without sedative side-effects on the central nervous system, due to favourable physicochemical properties. Its low lipophilicity and its marked hydrogen-bonding ability prevent its penetration through the blood/brain barrier [2, 3]. This was confirmed by a low rate of side-effects and unchanged psychological tests after administration of epinastine in clinical studies [4, 5].

Receptor-binding studies have indeed demonstrated that epinastine binds the H₁ receptor with a high affinity and selectivity. Epinastine also binds the α -adrenergic and the 5-HT₂ receptor with somewhat weaker binding affinity, but there was only poor binding to muscarinic receptors [6]. The antihistamine properties of epinastine have been demonstrated in several studies. Epinastine inhibits histamine-induced contraction in guinea-pig ileum [6, 7]. Epinastine also significantly reduces the histamine skin wheal size in rats and dogs [6], as well as in human volunteers [4]. Oral administration of epinastine prevents

the bronchoconstriction to inhalation and intravenous administration of histamine in guinea-pig [6, 8–10]. A bronchospasmolytic effect of epinastine after histamine inhalation has also been demonstrated in man [5]. FÜGNER *et al.* [6] and TASAKA and co-workers [7] observed the inhibition of histamine release from rat peritoneal mast cells *in vitro* by epinastine. MISAWA and co-workers [11] also found that chronic epinastine administration significantly inhibited the repeated antigen challenge-induced airway hyperresponsiveness to acetylcholine in rats.

Other properties of this drug are less well characterized. Pharmacological studies have identified epinastine as a 5-HT₂ antagonist, probably due to its structural resemblance to other guanidines which have been shown to be peripherally acting 5-HT₂ antagonists [1]. Epinastine has been shown to block the serotonin (5-HT)-induced oedema in rat paw [6], and it also inhibited granulocyte infiltration in guinea-pig respiratory and dermal tissue, which suggests an anti-inflammatory activity, possibly contributing to its clinical efficacy [12]. Bronchoconstriction to inhalation of 5-HT (in rats) and platelet-activating factor (PAF) (in guinea-pig) was significantly reduced by epinastine. This effect was more pronounced with

epinastine compared to ketotifen [8]. Intravenous administration of epinastine in anaesthetized guinea-pigs protected against bradykinin-induced bronchoconstriction. However, there was no effect of epinastine on the bronchoconstriction induced by endothelin-1, prostaglandin D₂, leukotriene D₄, substance P and neurokinin A [10]. The underlying mechanism of the inhibitory effect of epinastine on bradykinin-, 5-HT- and PAF-induced bronchoconstriction has not been elucidated.

In guinea-pig lower trachea and bronchi, electrical field stimulation (EFS) results in a biphasic contraction, consisting of a rapid and transient cholinergic component and a longer lasting nonadrenergic, noncholinergic (NANC) component [13]. This is due to the release of neuropeptides from sensory nerves, since it can be blocked by tachykinin receptor antagonists [14, 15], as well as by pretreating the animal with capsaicin, known to destroy the sensory nerves containing tachykinins [16].

Recently, we and others have demonstrated that serotonin modulates the noncholinergic contraction in guinea-pig airways *in vitro* by a prejunctional 5-HT₁ like receptor [17, 18]. We have also demonstrated that ketotifen, an antihistamine drug with 5-HT antagonistic properties, inhibits noncholinergic contraction, which was explained by stimulation of a prejunctional 5-HT₁-like receptor. As ketotifen was also known as a 5-HT antagonist, this effect could only be explained by assuming that ketotifen acts as a partial 5-HT agonist at higher concentrations [19]. In the present study, we investigated whether epinastine could modulate the excitatory NANC (eNANC) contraction in guinea-pig airways *in vitro*, and furthermore, we tried to identify the receptor responsible.

Methods

Tissue preparation

Dunkin-Hartley guinea-pigs of either sex (300–500 g) were killed by cervical dislocation. The lungs, with the bronchi and the trachea, were rapidly removed and placed in Krebs-Henseleit solution of the following composition (mMol·L⁻¹): NaCl 118; MgSO₄ 1.2; KCl 5.9; CaCl₂ 2.5; NaH₂PO₄ 1.2; NaHCO₃ 25.5; and glucose 5.05. The trachea was carefully stripped of connective tissue, opened longitudinally by cutting through cartilage, and cut into segments containing 4–5 cartilaginous rings. The main bronchi were dissected and ring preparations, 2–3 mm in length, were made. The tissues, connected to steel hooks both at the bottom of the 10 mL organ baths and at the force-displacement transducer, were mounted between two parallel platinum wire electrodes. The 10 mL organ baths contained Krebs-Henseleit solution, maintained at 37°C, and continuously bubbled with 5% CO₂ in O₂, giving a pH of 7.4. A resting tone of 1 g for the tracheal strips and 0.5 g for the bronchial ring segments was applied, which was found to be optimal for measuring changes in tension.

The tissues were then allowed to equilibrate for 1 h. During that time they were washed with fresh Krebs-Henseleit solution every 20 min. All experiments were performed in the presence of atropine (1 µM) to inhibit the rapid cholinergic contraction, and in the presence of

indomethacin (10 µM) to prevent modulation of neural responses by endogenously synthesized prostaglandins [20]. Propranolol was not present as it is an antagonist of 5-HT₁ receptors [21].

Protocol

The experimental protocol was identical for bronchial rings and for tracheal strips.

Electrical field stimulation (EFS). EFS was performed with a Harvard student stimulator (Harvard Apparatus Ltd, Edenbridge, Kent, UK). Isometric contractile responses were measured by using a Grass FT 03 force-displacement transducer (Stag Instruments Ltd, Chalgrove, Oxon, UK). The traces were visualized on a computer screen after digitalization of the signal (Codas; Dataq Instrument Inc., Akron, Oh, USA) and recorded on a personal computer. Biphasic square-wave pulses were delivered for 20 s periods, using a supramaximal voltage of 50 V at source, a pulse duration of 0.5 ms and a frequency of 8 Hz. This frequency has been shown to elicit measurable, reproducible NANC responses, that were ±50% of the maximal NANC response and have also been found to be optimal for detection of modulatory effects [22]. A frequency-response was not performed because repeated stimulations in the same tissues gave inconsistent responses.

After a 1 h equilibration period, a first stimulus was delivered in lower tracheal and bronchial segments. This produced a rapid atropine-sensitive contraction, followed by a longer lasting atropine-resistant NANC contraction. Stimuli were delivered every 20–30 min or when the tension had returned to baseline levels. Atropine (1 µM) was added 10 min before the second stimulation. Two control stimuli were then delivered. If the contractile responses were not consistent (*i.e.* >10% variation), the tissues were discarded. Epinastine (0.1–100 µM) was then added to the organ bath, with only one concentration of drug added per tissue. After a 15 min incubation period, a further two stimuli were delivered. Preliminary experiments involving a time course of inhibitory effect of epinastine demonstrated a maximum inhibition of the eNANC contraction after a 15 min incubation period, with no further inhibition with longer incubation time. The responses to EFS in control tissues were stable throughout the period of the experiment.

In a different set of experiments, after the two control stimulations, the tissues were pretreated either with methiothepin (0.1 µM), methysergide (1 µM), ketanserin (10 µM), tropisetron (1 µM), phentolamine (10 µM), or thioperamide (10 µM) each for 10 min. Subsequently, epinastine was added, and the same protocol was used as described above. Control tissues were treated with the antagonists only. In another set of experiments, the effect of chlorpheniramine (10 µM) was investigated in exactly the same way as epinastine.

Contractile responses evoked by EFS were completely abolished by tetrodotoxin (3 µM), confirming their neural origin. The eNANC responses could also be abolished by pretreatment of the guinea-pig with capsaicin [17], confirming that they were due to the release of neuropeptides from C-fibres.

Cumulative concentration-response relationship to exogenously applied substance P. To determine whether the inhibitory effect of epinastine was due to activation of pre- or postjunctional receptors, the effect of epinastine (100 μM) on the cumulative concentration-response relationship to exogenously applied substance P (0.01–10 μM) after a 15 min incubation period was studied. The results were expressed as a percentage of the maximum contraction to acetylcholine (10 mM), which was determined at the beginning of the experiment.

Drugs

Drugs used in these experiments were obtained from the following sources: epinastine (Boehringer Ingelheim KG, Ingelheim am Rhein, Germany); substance P, indomethacin, atropine sulphate, acetylcholine, ketanserin, chlorpheniramine (Sigma Chemical Co., Filter Service, Eupen, Belgium); methiothepin maleate, phentolamine, thioperamide (RBI, Sanver Tech, Boechout, Belgium); methysergide and tropisetron (ICS 205-930) (a kind gift from Sandoz, Basel). Indomethacin was dissolved in alkaline phosphate buffer (pH 7.8) composed of 20 mM KH_2PO_4 and 120 mM Na_2HPO_4 . Ketanserin and tropisetron were dissolved in dimethylsulphoxide. All other drugs were dissolved in distilled water. Fresh drug solutions were made up daily and diluted with distilled water. Where appropriate, control tissues were treated either with distilled water or with the specific solvent. Drug additions did not exceed 1% (v/v) of the organ bath volumes. All concentrations refer to the final bath concentration.

Analysis of results

Results are expressed as mean \pm SEM. All contractile responses, measured as the difference between peak tension and resting tension that developed, were expressed in absolute changes in tension and then transformed to a mean response for the two control stimulations in each tissue. The effect of a single concentration of epinastine or chlorpheniramine, with or without antagonist, was expressed as a percentage inhibition, also using the mean of two stimulations. Significance was assessed using a Student's *t*-test for paired or unpaired data. The same test was used to assess the effect of epinastine or ketotifen on the cumulative dose-response curve to exogenous substance P. Probability values of less than 0.05 were considered significant. The concentrations required to produce a half maximal effect (EC_{50}) were calculated by iterative curve fitting using Graphpad Prism (Graphpad Software Inc., San Diego, CA, USA). However, these values were obtained only for the mean curve data, as only one concentration of agonist was tested per tissue. Therefore, it was not possible to calculate SEM or to statistically compare the EC_{50} values obtained.

Results

The results obtained in trachea and main bronchi were comparable and, therefore, taken together.

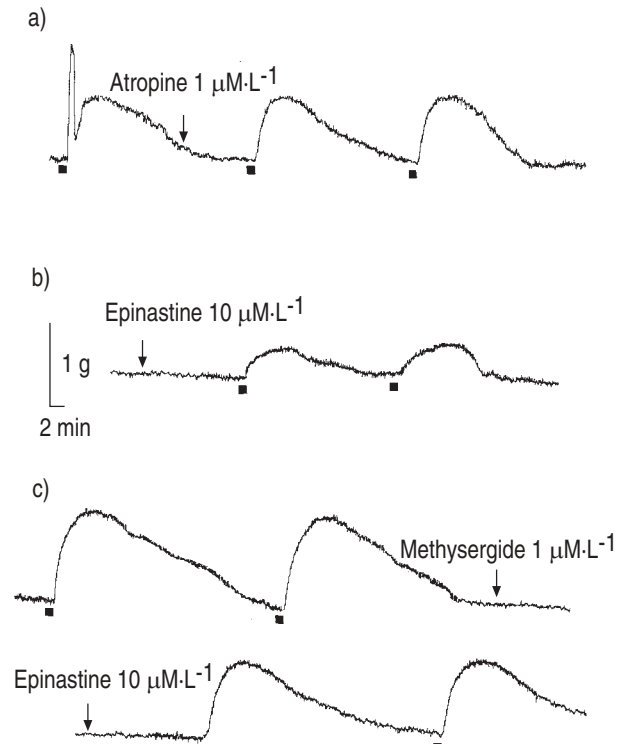


Fig. 1. — a) Tracing showing the effect of epinastine on the NANC contraction elicited by EFS (50 V at source, 0.5 ms, 8 Hz for 20 s; represented by ■) in guinea-pig lower trachea, before and after atropine (1 μM). b) Epinastine (10 μM) produces 55% inhibition. c) In another tissue of the same animal, the epinastine (10 μM) induced inhibition of the NANC contraction is only 20% in the presence of methysergide 1 μM . NANC: nonadrenergic noncholinergic; EFS: electrical field stimulation.

Effect of epinastine on the eNANC response

EFS in guinea-pig lower trachea and main bronchus results in a rapid and transient cholinergic contraction and a longer-lasting eNANC contraction due to neuropeptide release from sensory nerves. A typical trace of the protocol used is shown in figure 1. This figure also demonstrates the effect of epinastine (10 μM) on lower trachea after a 15 min incubation period. There is no significant difference between the first and second stimulus (after 20–30 min incubation), which proves that a 15 min incubation period is sufficient. Methysergide (1 μM) clearly attenuates the epinastine (10 μM) induced inhibition of the eNANC contraction.

Epinastine produced a concentration-dependent inhibition of the eNANC neural contractile response to EFS at 8 Hz, with a maximum inhibition of $91 \pm 7\%$ at a concentration of 100 μM ($n=5$; $p<0.001$) (fig. 2) and an EC_{50} value of 9.9 μM .

Effect of chlorpheniramine on the eNANC response

Chlorpheniramine (10 μM) failed to produce a significant inhibition of the eNANC contraction ($10 \pm 6\%$ inhibition) ($n=6$; ns) (data not shown).

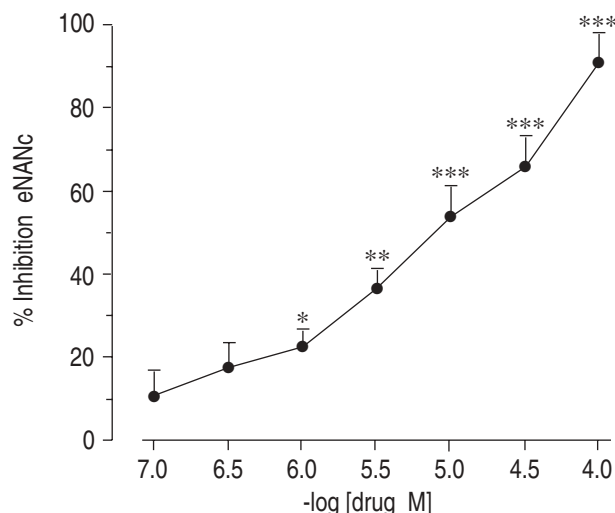


Fig. 2. — The effect of epinastine on the eNANC contraction elicited by EFS (50 V at source, 0.5 ms, 8 Hz for 20 s) in guinea-pig airways *in vitro*. Atropine (1 μ M) was present throughout. Epinastine (—●—) produces a concentration-dependent inhibition, with an EC₅₀ value of 9.9 μ M. Points represent mean \pm SEM of at least n=5 observations. Significance of inhibition; *: p<0.05; **: p<0.01; ***: p<0.001, compared to control. EC₅₀: concentration required to produce a half maximal effect, eNANC: excitatory nonadrenergic noncholinergic. For further definitions see legend to figure 1.

Effect of 5-HT antagonists on the epinastine-induced inhibition of the eNANC contraction

Addition of ketanserin at a concentration that has been demonstrated to block 5-HT₂ receptors (10 μ M) [23] had no effect either on the eNANC response or on the inhibition of the eNANC contraction produced by epinastine (0.1–100 μ M) (fig. 3).

The nonselective 5-HT₁/5-HT₂ antagonists, methysergide (1 μ M) and methiothepin (0.1 μ M), had no inhibitory

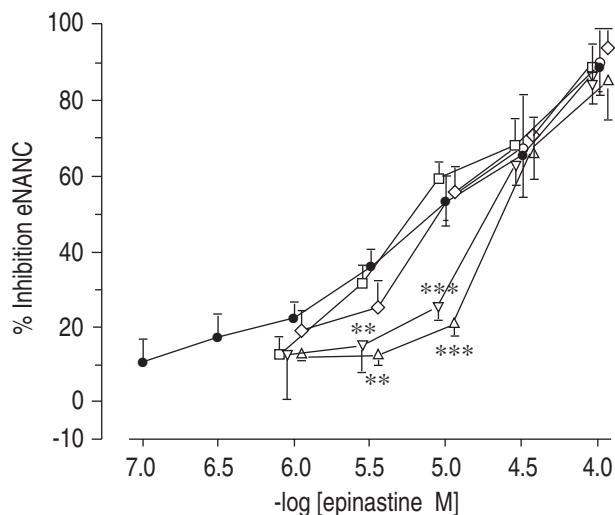


Fig. 3. — Inhibitory effect of epinastine, 0.1–100 μ M (—●—), on the eNANC contraction elicited by EFS (50 V at source, 0.5 ms, 8 Hz for 20 s) in guinea-pig airways *in vitro*, and attenuation of this inhibition by methiothepin, 0.1 μ M (—▽—), and methysergide, 1 μ M (—△—). Ketanserin, 10 μ M (—○—), and tropisetron, 1 μ M (—◇—), on the other hand, had no effect on the NANC inhibition produced by epinastine. Points represent mean \pm SEM of at least 5 observations. Significance of inhibition; **: p<0.01; ***: p<0.001, compared to epinastine alone. For definitions see legends to figures 1 and 2.

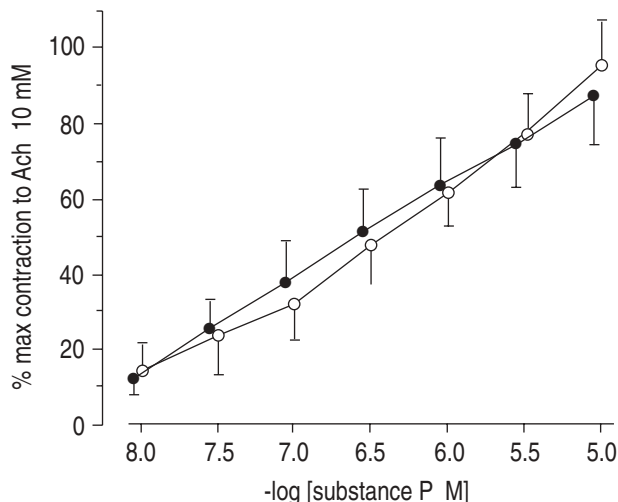


Fig. 4. — Effect of epinastine (100 μ M) on the cumulative concentration-response relationship to exogenously applied substance P (10 nM to 10 μ M). Effects are displayed as a percentage of the maximum (max) contraction to acetylcholine (10 mM). Curves are shown for substance P in the absence (—○—) and in the presence of epinastine (—●—). Points represent mean \pm SEM of five observations. ACh: acetylcholine.

effect on the NANC contractile response (0.4 \pm 2.3% and 4.8 \pm 2.7% inhibition, respectively; n=6–11) (NS compared to control). However, both antagonists attenuated the inhibitory effect of epinastine (0.1–100 μ M). On the other hand, methysergide and methiothepin did not reduce the maximum response to epinastine, suggesting a competitive blockade of the effect of epinastine by both antagonists (fig. 3).

Tropisetron (ICS 205-930), a 5-HT₃ and 5-HT₄ antagonist (1 μ M), had no effect on the eNANC response and did not modulate the concentration-dependent inhibition of the eNANC contraction by epinastine (0.1–100 μ M) (fig. 3).

Effect of phentolamine and thioperamide on the epinastine-induced inhibition of the eNANC contraction

Phentolamine (1 μ M), an α -adrenergic antagonist, failed to prevent the inhibition of the eNANC contraction by epinastine (data not shown).

Thioperamide (10 μ M), a histamine H₃ antagonist, also failed to prevent the inhibition of the eNANC contraction by epinastine (data not shown).

Effect of epinastine on the cumulative concentration-response relationship to exogenously applied substance P

Pretreatment with epinastine (100 μ M) did not significantly alter the response to substance P (0.01–10 μ M) in guinea-pig airways (n=5; NS) (fig. 4).

Discussion

We have demonstrated that epinastine inhibits the eNANC neural contraction elicited by EFS in guinea-pig

airways *in vitro*. Indeed, epinastine produced a concentration-dependent inhibition of the eNANC contraction with a maximum inhibition of about 90% at 100 μ M. Moreover, epinastine did not affect the cumulative concentration-response relationship to exogenous substance P, suggesting that the inhibitory effect of epinastine is exerted through a prejunctional mechanism.

As demonstrated by VERLEDEN *et al.* [19] and KAMIKAWA [24], ketotifen also inhibits the NANC contraction in guinea-pig bronchi and trachea by a prejunctional mechanism. Compared to our results, we found that the inhibitory effect of ketotifen is less pronounced than the inhibition by epinastine, with epinastine being about three-fold more potent than ketotifen. Epinastine is an antihistaminic drug that has some affinity to α -adrenergic and 5-HT receptors [6]. Therefore, the aim of this study was also to investigate which prejunctional receptor was responsible for the inhibitory effect of epinastine on the NANC neurotransmission in guinea-pig airways.

Chlorpheniramine, a histamine H₁ receptor antagonist, did not produce any inhibition of the NANC contraction, which virtually excludes the possibility of prejunctional histamine H₁ receptor involvement. This is in agreement with our previous results, in which it was demonstrated that cetirizine, a very potent and specific histamine H₁ receptor antagonist had absolutely no effect on the noncholinergic contraction [19]. As epinastine has a low binding affinity at histamine H₂ receptors and as histamine agonistic properties have not been described, it seemed very unlikely that a histamine H₂ mechanism could account for the inhibition of the NANC contraction. On the other hand, as histamine H₃ receptors modulate NANC bronchoconstriction in guinea-pig airways, one could assume that epinastine might possess H₃-agonistic properties. However, thioperamide, a selective H₃-antagonist failed to attenuate the inhibitory effect of epinastine. We therefore assumed that the epinastine-induced inhibition of the noncholinergic contraction could not be explained by an effect on histamine H₁ or H₃ receptors. The observation that epinastine inhibited the NANC response to a significantly greater degree than ketotifen, although ketotifen has a stronger histamine receptor-binding affinity, further corroborates this conclusion of modulation by nonhistamine receptor activation.

Epinastine also has a weak affinity at adrenergic receptors and α -adrenergic agonists modulate NANC contraction in guinea-pig airways [25]. Phentolamine, an α -adrenergic receptor antagonist failed, however, to prevent the inhibition produced by epinastine, which also excludes α -adrenergic agonistic activity as the possible mechanism of action of epinastine in inhibiting the eNANC contraction.

One could also argue that epinastine might modulate the noncholinergic contraction in guinea-pig airways *in vitro* by increasing the inhibitory nonadrenergic relaxation, which is due to the release of vasoactive intestinal peptide and nitric oxide, both potent smooth muscle relaxants [26, 27]. This is unlikely to be the explanation, however, since epinastine also modulated the noncholinergic contraction in bronchi, where a functional inhibitory NANC innervation has not been demonstrated [13, 28].

It has been established that 5-HT modulates the NANC

contraction in guinea-pig airways *in vitro* [17, 18]. Since epinastine has an affinity at 5-HT receptors [6], this could be an appropriate explanation for its effect on the eNANC contraction. Epinastine significantly prevents the bronchoconstriction to 5-HT inhalation in rats *in vivo* [8], and pharmacological studies have identified the drug as a 5-HT antagonist. However, in the present study, we hypothesized that epinastine acts as a 5-HT agonist and, indeed, we were able to show that methiothepin and methysergide (both 5-HT₁/5-HT₂ antagonists), used at a concentration that had no effect on the NANC contraction, could attenuate the inhibitory effect of epinastine on the EFS-induced noncholinergic contraction. Methysergide and methiothepin, however, did not reduce the maximum response to epinastine, suggesting a competitive blockade of the effect of epinastine by both 5-HT₁/5-HT₂ antagonists, which is consistent with epinastine acting at a 5-HT₁ receptor [29]. Furthermore, the inhibitory effect of epinastine could not be blocked by ketanserin, a 5-HT₂ antagonist, nor by tropisetron, a 5-HT₃/5-HT₄ antagonist.

Our results are consistent with previous studies, which have demonstrated the presence of a 5-HT receptor, located on sensory nerve endings [17, 18]. Because of difficulties in further defining the exact 5-HT receptor subtype, due to the lack of selective agonists and antagonists, we suggest that the 5-HT receptor involved in our study is of the 5-HT₁-like subtype, according to the criteria of BRADLEY *et al.* [29], which include: 1) susceptibility to antagonism by methiothepin and/or methysergide; 2) ineffectiveness of other 5-HT antagonists, such as ketanserin and tropisetron; and 3) ability of 5-CT (5-carboxamidotryptamine) to mimic the effect of 5-HT [29]. The first two criteria are fulfilled in the present study; whereas, WARD *et al.* [18] and PYPE *et al.* [17] have already demonstrated that 5-CT can mimic the effect of 5-HT in guinea-pig airways. As a consequence, we suggest that epinastine may modulate the release of neuropeptides by stimulation of a prejunctional 5-HT₁-like receptor, probably located to sensory nerve endings.

Since the release of tachykinins from airway sensory nerves by means of axon reflex mechanisms may be important in sustaining the inflammatory response in asthmatic airways [30], then epinastine, which inhibits the release of neuropeptides by activation of a prejunctional 5-HT receptor, may exert an effect in asthma by reducing this neurogenic component of airway inflammation. Preliminary clinical studies with epinastine suggest a significant improvement in asthma symptom control with epinastine [31]. As the efficacy of selective histamine H₁ antagonists in the treatment of asthma has not been established [32], it seems logical to assume that other mechanisms of action account for the prophylactic effect of epinastine. Therefore, the identification of epinastine as a 5-HT₁ agonist warrants its further study as a potential drug for asthma treatment.

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