

ORIGINAL ARTICLE

Role of 5-HT_{2A}, 5-HT₄ and 5-HT₇ receptors in the antigen-induced airway hyperresponsiveness in guinea-pigs

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Summary

Background A possible role of 5-hydroxytryptamine (5-HT) in the origin of antigen-induced airway hyperresponsiveness (AI-AHR) has been scarcely investigated.

Objective To explore the participation of different 5-HT receptors in the development of AI-AHR in guinea-pigs.

Methods Lung resistance was measured in anaesthetized guinea-pigs sensitized to ovalbumin (OVA). Dose–response curves to intravenous (i.v.) acetylcholine (ACh) were performed before and 1 h after antigenic challenge and expressed as the 200% provocative dose (PD₂₀₀). Organ bath experiments, confocal microscopy and RT-PCR were additionally used. The 5-HT content in lung homogenates was measured by HPLC.

Results Antigenic challenge significantly decreased PD₂₀₀, indicating the development of AI-AHR. This hyperresponsiveness was abolished by a combination of methiothepin (5-HT₁/5-HT₂/5-HT₅/5-HT₆/5-HT₇ receptors antagonist) and tropisetron (5-HT₃/5-HT₄ antagonist). Other 5-HT receptor antagonists showed three different patterns of response. Firstly, WAY 100135 (5-HT_{1A} antagonist) and ondansetron (5-HT₃ antagonist) did not modify the AI-AHR. Secondly, SB269970 (5-HT₇ antagonist), GR113808 (5-HT₄ antagonist), tropisetron or methiothepin abolished the AI-AHR. Thirdly, ketanserin (5-HT_{2A} antagonist) produced airway hyperresponsiveness. Animals with bilateral vagotomy did not develop AI-AHR. Experiments in tracheal rings showed that pre-incubation with LP44 or cisapride (agonists of 5-HT₇ and 5-HT₄ receptors, respectively) induced a significant increase of the cholinergic contractile response to the electrical field stimulation. In sensitized lung parenchyma strips, ketanserin diminished the contractile responses to ACh. Sensitization was associated with a ninefold increase in the 5-HT content of lung homogenates. Confocal microscopy showed that sensitization enhanced the immunolabelling and co-localization of nicotinic receptor and 5-HT in airway epithelium, probably located in pulmonary neuroendocrine cells (PNECs). RT-PCR demonstrated that neither sensitization nor antigen challenge modified the 5-HT_{2A} receptor mRNA levels.

Conclusions Our results suggested that 5-HT was involved in the development of AI-AHR to ACh in guinea-pigs. Specifically, 5-HT_{2A}, 5-HT₄ and 5-HT₇ receptors seem to be particularly involved in this phenomenon. Participation of 5-HT might probably be favoured by the enhancement of the PNECs 5-HT content observed after sensitization.

Keywords 5-HT, acetylcholine, airway hyperresponsiveness, airway smooth muscle, neuroendocrine cells, serotonin

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Introduction

5-Hydroxytryptamine (5-HT or serotonin) is an important neurotransmitter of the central and peripheral nervous systems, and can also be synthesized by non-neuronal sources such as intestinal enterochromaffin cells and pulmonary neuroendocrine cells (PNECs). 5-HT actions in the organism are multiple and complex, primarily due to the large number of receptor subtypes. Seven serotonin receptors have been identified (5-HT₁ to 5-HT₇), many of them further divided into subcategories.

In the respiratory system of different mammalian species, including humans, 5-HT is localized in airway mast cells and neuroendocrine cells [1–3]. 5-HT levels in pulmonary tissues are directly proportional to their plasmatic concentration, the platelets being its main source [4].

The role of 5-HT in asthma has been controversial. Plasma concentrations of free 5-HT in asthmatic subjects notably increase during an asthmatic exacerbation and this increment is related to asthma severity [5]. In addition, it has been reported that tianeptine, a drug that lowers plasma 5-HT by enhancing the 5-HT re-uptake, improves pulmonary function in asthmatic children [6]. Other evidences have shown that 5-HT promotes migration and accumulation of mast cell and eosinophils [7, 8]. These observations suggest that 5-HT might be highly involved in the pathogenesis of chronic asthma and/or asthma exacerbations.

The role of 5-HT in the development of antigen-induced airway hyperresponsiveness (AI-AHR) has scarcely been investigated. In animal models, the only 5-HT receptor claimed to be involved in the development of airway hyperresponsiveness (AHR) has been the 5-HT_{2A} receptor [9]. This receptor subtype may also be responsible for allergy-induced pulmonary eosinophilia in a mice model [8]. In addition, it has recently been demonstrated that 5-HT₂ receptors facilitate acetylcholine (ACh) release from parasympathetic cholinergic neurons in sensitized mice [10]. Thus, the aim of the present work was to explore the possible role of different 5-HT receptors in the AI-AHR in guinea-pigs.

Materials and methods

We used male Hartley guinea-pigs (450–500 g) bred under conventional conditions in our institutional laboratory-animal facilities (12/12 h light/dark cycles, filtered conditioned air, 21±1 °C, 50%–70% humidity, sterilized bed) and fed *ad libitum* with pellets (Teklad Guinea Pig Diet 7006; Harlan Teklad, Madison, WI, USA) and sterilized water. The protocol was approved by the Scientific and Bioethics Committees of the Instituto Nacional de Enfermedades Respiratorias. Experiments were conducted in accordance with the published Guiding Principles in the Care and Use of Animals approved by the American Physiological Society.

Most studies were performed using guinea-pigs submitted to one of three experimental conditions: guinea-pigs sensitized to ovalbumin (OVA) and studied shortly after antigenic challenge (sensitized+antigenic challenged group), guinea-pigs sensitized to OVA (sensitized group) and non-sensitized guinea-pigs (controls).

Sensitization procedure and antigenic challenge

Guinea-pigs were sensitized at day 0 by an intraperitoneal (i.p.) administration of 60 mg OVA and 1 mg Al(OH)₃ in 0.5 mL saline (0.9% NaCl). At day 8, animals were nebulized with 3 mg/mL OVA in saline for 5 min, delivered by an ultrasonic nebulizer (model US-1; Puritan Bennett, Carlsbad, CA, USA). Guinea-pigs were nebulized again on day 15 with 0.5 mg/mL OVA in saline for 1 min. On the day of the study (day 21–25), the animals were subjected to plethysmography in order to perform a first measurement of airway responsiveness to ACh, which was followed by an antigenic challenge with a single bolus of OVA (400 µg/kg i.v.), and then by a second airway responsiveness measurement 1 h after recovering from the antigenic challenge. Exceptional guinea-pigs with a poor response to antigen were excluded from the study.

Plethysmography for mechanically ventilated animals

Lung resistance (R_L) was measured using the isovolumetric method in a closed-chamber plethysmograph (Buxco Electronics Inc., Troy, NY, USA). Guinea-pigs were anaesthetized with pentobarbital sodium (35 mg/kg i.p.), and the depth of anaesthesia was maintained with the hourly administration of additional doses of pentobarbital (~9 mg/kg i.v.). Each animal received pancuronium bromide (0.06 mg/kg i.v.) to avoid spontaneous respiratory movements. After the trachea was cannulated, each animal was mechanically ventilated (model 50–1700 ventilator; Harvard Apparatus Ltd., Edenbridge, UK) with a tidal volume of 10 mL/kg and 48 breaths/min. The right jugular vein and the left carotid artery were cannulated for drug administration and for arterial pressure recording (R-612 dynograph; Beckman, Schiller Park, IL, USA), respectively. A water-filled cannula was positioned into the middle third of the oesophagus to measure intraoesophageal pressure as a surrogate of intrapleural pressure. Pressures obtained from the oesophageal and tracheal cannulas were recorded using a differential pressure transducer (SCXL004DN; SenSym ICT, Milpitas, CA, USA). Pressure inside the plethysmograph chamber was also recorded using a differential pressure transducer. This last signal was converted to a pseudoflow signal using a software (Buxco Biosystem XA, version 1.0). Finally, this software calculated the relationship between both parameters to obtain R_L using the formula $R_L = \Delta P / \Delta \dot{V}$, where ΔP is the pressure change and $\Delta \dot{V}$ is the flow change.

Airway responsiveness measurement

After a baseline response to saline (0.1 mL i.v.), a dose-response curve to ACh (1, 1.8, 3.2, 5.6, 10, 18, 32, 56 and 100 µg/kg i.v.) was constructed before and after the antigenic challenge with OVA. After each ACh dose, the maximum response (increment of R_L) obtained at any moment during the following 3 min was registered. The curve was finished when the response to an ACh dose reached threefold the baseline R_L value. Airway responsiveness was expressed as the provocative dose 200% (PD₂₀₀, i.e. the ACh dose required to obtain a 200% increment of the basal R_L value).

Ten minutes before the second dose-response curve to ACh, vehicle or different antiserotonergic drugs were administered i.v. (Table 1). In order to ensure that antigenic challenge always produced AHR, a control group of four to seven sensitized animals without antiserotonergic treatment (i.e. vehicle administration) were simultaneously studied along with almost every experimental group. We corroborated that antiserotonergic drugs did not change the basal R_L values.

In another set of experiments, after guinea-pigs were recovered from the antigenic challenge (~15 min before the second dose-response curve to ACh), both vagal nerves were dissected using glass rods, tied with a silk thread and cut. Control (sham) animals for these experiments were only subjected to vagal dissection, but not to vagotomy.

Tracheal rings

Guinea-pigs sensitized to OVA were deeply anaesthetized with pentobarbital sodium (35 mg/kg, i.p.) and exsanguinated. The major airways were carefully dissected and cleaned of connective tissue, and four rings were obtained from the middle of the trachea (each ring was subjected to different experimental conditions). Each tracheal ring was hung in a 10 mL organ bath between two hooks inserted into the lumen. One of the hooks was attached to an

isometric transducer (FT03; Grass Instruments, West Warwick, RI, USA) by a 4-0 silk thread. The other hook acted as an anchor by keeping the tracheal ring fixed to a Plexiglas rod. The composition of Krebs solution in the organ baths was (mM): NaCl 120, KCl 4.77, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, CaCl₂ 2.5 and glucose 11, which was maintained at 37 °C and bubbled with 5% CO₂ in oxygen (pH 7.4). Tissues were placed under a resting tension of 1 g for 30 min, and washed with fresh Krebs at 15-min intervals meanwhile. Isometric transducers were connected to a preamplifier (Cyber Amp 380, Axon Instruments Inc., Foster City, CA, USA), signals were digitized with an interface (Digidata 1200, Axon Instruments Inc.) and tension changes were continuously monitored using a software (AxoScope v9.0, Axon Instruments Inc.) in a computer.

Tissues were stimulated three times with KCl (60 mM), followed by an electrical field stimulation (16 Hz, 2 ms and 100 V for 10 s) through platinum electrodes. Exceptional rings with a poor response to these stimuli were excluded from the study. Afterwards, two additional electrical field stimulations (0.5 Hz) were provided 20–30 min apart, administering either 2.56 µM LP44 (a high-affinity 5-HT₇ receptor agonist [26]) or 1.0 µM cispripide (a 5-HT₄ receptor agonist [15, 27]) 15 min before the second field stimulation. In these experiments, only the initial, fast contractile response induced by the electrical field stimulation was taken into account. This response is considered to be cholinergic in nature because it is completely abolished by atropine [28]. We corroborated that two sequential stimulations with 0.5 Hz produced a similar contractile response of tracheal rings.

Lung parenchyma strips

Guinea-pigs were anaesthetized and mechanically ventilated as described above. In the case of the sensitized + antigenic challenged group, animals were killed approximately 2 h after the antigen administration.

Table 1. Antiserotonergic drugs used in the study

5-HT antagonist	Subtype of 5-HT receptor antagonized	<i>n</i>	Dose (mg/kg)	References
GR113808	4	7	1.0	Kojima et al. [11] and Gale et al. [12]
Ketanserin	2A	8	1.0	Campos-Bedolla et al. [13] and Buckner et al. [14]
Methiothepin	1, 2, 5, 6, 7	6	0.3	Gerhardt and van Heerikhuizen [15] and Roberts et al. [16]
Ondansetron	3	7	1.0	Singh et al. [17] and Youssefyeh et al. [18]
SB269970	7	5	3.0	Thomas et al. [19] and Guscott et al. [20]
Tropisetron	3, 4	5	3.0	Villalon et al. [21], Schiavi et al. [22] and Wang et al. [23]
WAY100135	1A	6	10.0	Mundey et al. [24] and Assié et al. [25]
Methiothepin + Tropisetron	1, 2, 5, 6, 7 3, 4	6	0.3 3.0	Roberts et al. [16] Villalon et al. [21]

5-HT, 5-Hydroxytryptamine.

Control animals were killed at the same time period. After exsanguination, the lungs–heart block was exposed and intravascular blood was removed from the lungs by introducing 20 mL saline solution through the right ventricle. Then, the lungs were removed and eight parenchyma strips ($\sim 3 \times 3 \times 20$ mm, ~ 56 mg wet weight) were cut from the diaphragmatic edge of the lower lobes.

Every parenchymal preparation was suspended in a 10 mL organ bath containing Krebs solution. The lower end of the strip was attached to the hook of a Plexiglas rod with a 4-0 silk thread. The upper end was attached to an isometric transducer.

A cumulative concentration–response curve to ACh was performed (0.01–320 μ M at half-log intervals) in control and sensitized + antigenic challenged tissues. Some tissues were incubated with hexamethonium (30 μ M) or ketanserin (10 nM). All responses were normalized to 60 mM KCl response.

Immunohistochemical study

Sensitized and non-sensitized guinea-pigs were deeply anaesthetized with pentobarbital sodium (35 mg/kg, i.p.) and exsanguinated. Lung tissues were fixed in 4% paraformaldehyde (PFA) for 2.5 h and then placed in ethanol (30%) until their inclusion in paraffin. Once paraffinized, tissue blocks were cut into thin slices (5 μ m) and placed over silane-prep slides (Sigma Chem. Co., St. Louis, MO, USA).

Paraffin was removed by incubation in xylol, followed by graded alcohols. Lung slices were placed in phosphate-buffered saline (PBS), three times for 5 min each, followed by PBS with 0.25% Triton-X 100 (TX-100) for 15 min. To block non-specific binding to proteins, 10% normal goat serum was applied on the slices for 30 min. The primary antibody, rabbit polyclonal to mouse nicotinic ACh receptor $\alpha 7$ (Ab23832; Abcam, Cambridge, UK), was incubated (1 : 100) overnight at room temperature. The slices were rinsed with PBS-Tween 0.5% three times for 5 min each. The secondary antibody, a goat anti-rabbit conjugated with FITC (Cat # 81–6111; Invitrogen, Carlsbad, CA, USA), was incubated (1 : 100) for 1 h under dark conditions and then rinsed with PBS-Tween 0.5% three times for 5 min each. Afterwards, slices were fixed with 4% PFA for 15 min and rinsed with PBS.

To incubate the next primary antibody (mouse monoclonal antihuman 5-HT, MO758; Dako, Carpinteria, CA, USA), the above-described protocol was used, but using 10% normal horse serum to block non-specific binding to proteins, and donkey anti-mouse conjugated with Cy5 (Cat # 715-176-150; Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary antibody. The slices were covered by mounting medium with an anti-fading agent (Fluka, Sigma-Aldrich, Buchs SG, Switzerland) and coverglass was sealed with varnish. To deter-

mine the specificity of immunolabelling, the primary antibody was omitted.

The immunolabelled slices were observed using a confocal laser microscope (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany). The resolution of all images obtained with the confocal microscope using a $\times 40$ oil immersion objective was originally 1024 $\times$ 1024 pixels. For display purposes, merged images were constructed in which the nicotinic receptor was in green, 5-HT was in red and the good signal overlap (co-localization) was in yellow.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from lung tissue or tracheal smooth muscle using TRIzol reagent (Life Technologies, Grand Island, NY, USA). RNA quality was assessed by resolution on denatured 1% agarose gels and measurement of absorbance ratios at 260/280 nm. One microgram of total RNA was reverse transcribed using 2 μ g of random primers and Moloney murine leukaemia virus reverse transcriptase according to the manufacturer's protocol (Advantage RT-for-PCR Kit; Clontech, Palo Alto, CA, USA). Quantitative real-time PCR amplification was performed using the i-Cycler iQ Detection System (Bio-Rad, Hercules, CA, USA). PCR was performed with cDNA working mixture in a 25 μ L reaction volume containing 3 μ L of cDNA, 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μ M dNTP, 1 μ M specific 5' and 3' primers, 1.25 U of Taq DNA polymerase (Roche, Branchburg, NJ, USA) and 10 nM fluorescein and dye SYBR green I 1 : 50 000 (Roche, Indianapolis, IN, USA). A pair of primers for 5HT_{2A} receptor mRNA was designed in Primer BLAST, and the sequences were 5'-CTGCAGGATGATTCCAAGGT-3' and 5'-AAATGCCACGAAAGAACCAA-3'. A dynamic range was built with each product of PCR on copy number serial dilutions of 1×10^8 , 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 and 1×10^2 . All PCRs were performed in triplicate. Standard curves were calculated referring the threshold cycle (the PCR cycle at which a specific fluorescence becomes detectable) to the log of each cDNA dilution step. Results were expressed as the number of copies of the target gene normalized to 18S rRNA, which was quantified using the same conditions of PCR but using additionally a taqman probe cat. 4352930E (Applied Biosystems, Foster City, CA, USA).

5-hydroxytryptamine concentrations in lung homogenates

Heart–lungs blocks from control, sensitized and sensitized + antigenic challenged guinea-pigs were obtained as described in 'Lung parenchyma strips', except that the perfusate liquid contained 10^{-4} M phenelzine (a non-selective inhibitor of monoamine oxidases). Afterwards, the right diaphragmatic lobe was excised and stored at -70°C until its analysis.

5-HT concentrations in the lung homogenates were measured using the cation exchange HPLC-EC detection method using an improved method described elsewhere [29]. The isocratic analytical system consisted of an analytical column for monoamine HR-80 (ESA Inc., Chelmsford, MA, USA) and an electrochemical detector (Coulchem II; ESA Inc.) that were coupled to the HPLC (model 9012, Varian, Walnut Creek, CA, USA). The mobile phase (90 mM sodium dihydrogen phosphate, 50 mM citric acid, 1.7 mM 1-heptanesulphonate sodium salt, 50 mM EDTA, 10% acetonitrile, pH 3.0 adjusted with NaOH semiconductor grade) was pumped at a rate of 1 mL/min at 30 °C. Data were stored in a microcomputer and analysed using a data acquisition and analysis software (Star Chromatography Workstation v4.01; Varian). 5HT concentrations were adjusted by protein content (measured by Bradford's method), and expressed as pmol/mg protein.

Statistical analysis

In *in vivo* experiments, PD₂₀₀ to i.v. ACh was calculated for each dose-response curve by extrapolation in a linear regression analysis (CurveExpert v1.3; Daniel G. Hyams, Hixson, TN, USA). Change in the PD₂₀₀ before and after antigenic challenge was evaluated using paired Student's *t*-test. In tracheal rings, change in the contractile response to electrical field stimulation was evaluated using a one-sample *t*-test. Experiments in lung parenchyma strips were evaluated through the EC50 and maximum responses to ACh and analysed by paired Student's *t*-test with the Bonferroni correction for multiple comparisons. The 5-HT concentration in the lung homogenates was compared using ANOVA and Tukey's test. Data in the text and illustrations correspond to mean ± SEM. Statistical significance was set at two-tailed *P* < 0.05.

Drugs

Methiothepin mesylate, ketanserin tartrate, tropisetron hydrochloride, hexamethonium chloride and phenelzine were purchased from Sigma Chem. Co. GR113808, SB 269970 hydrochloride, WAY100135 dihydrochloride, LP44 [4-(2-methylthiophenyl)-*N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide] and cisapride were purchased from Tocris (Ellisville, MO, USA). Ondansetron was obtained from a commercial presentation (Zofran[®], GlaxoWellcome, Mexico City, Mexico). All drugs were diluted in saline solution.

Results

Taking into account all the animals studied (*n* = 88), the basal *R_L* value averaged 0.557 ± 0.014 cm H₂O/mL/s, and the antigenic challenge induced a drastic increase of the

R_L up to 2.145 ± 0.134 cm H₂O/mL/s, which returned to near-basal values after approximately 15–30 min. One hour after recovering from this acute response, the antigenic challenge produced AHR to ACh in every control group. This increased responsiveness was evident because PD₂₀₀ values from the second dose-response curves (after OVA challenge) were significantly lower than those obtained in the first ones (before OVA challenge). This change is exemplified in Fig. 1a, where the average PD₂₀₀ values of the first and the second dose-response curves were 14.1 ± 2.3 and 9.8 ± 2.2 µg/kg, respectively (i.e. PD₂₀₀ values of second curves corresponded to 66.3 ± 6.6% of the PD₂₀₀ from first ones, *n* = 7, *P* < 0.002). We corroborated that AHR was not observed in sensitized animals in the absence of acute antigenic challenge, because the second curves corresponded to 141.1 ± 37.5% of the first ones (*n* = 4, *P* = 0.35, not illustrated). Simultaneous antagonism of all 5-HT receptors through a combination of methiothepin plus tropisetron completely abolished this AHR (15.9 ± 1.2 vs. 28.8 ± 8.5 µg/kg, 174.1 ± 41.8%, *n* = 6, *P* = 0.16, Fig. 1b). In order to identify the subtypes of 5-HT receptors involved in this effect, several antiserotonergic drugs were also evaluated separately, and at least three different patterns of response were observed (Fig. 2). Firstly, WAY100135 and ondansetron did not modify the AI-AHR, as PD₂₀₀ values were still lower after antigenic challenge (60.3 ± 9.4%, *P* = 0.002, in the WAY100135 group and 73.8 ± 4.6%, *P* = 0.0001, in the ondansetron group). Secondly, SB269970, GR113808, tropisetron or methiothepin abolished the AHR, as PD₂₀₀ values before and after antigen challenge were not different with any of these drugs (100.2 ± 20.3%, 101.8 ± 12.3%, 110.1 ± 14.9% and 127.0 ± 20.5%, respectively). Thirdly, ketanserin pre-incubation led to even higher PD₂₀₀ values after antigenic challenge than before OVA (237.4 ± 62.6%, *P* = 0.04), i.e. ketanserin produced hyporesponsiveness. In non-sensitized guinea-pigs, we corroborated that ketanserin did not modify the dose-response curve to ACh (18.4 ± 4.5 vs. 21.9 ± 8.5 µg/kg, i.e. 105.96 ± 25.14%; *n* = 4, *P* = 0.45).

In order to evaluate the role of vagal nerves in the development of AI-AHR to ACh, a group of guinea-pigs were subjected to bilateral vagotomy before the second dose-response curve. In these experiments, we found that vagal sectioning completely abolished the AI-AHR, as no difference was seen in the PD₂₀₀ values before and after antigenic challenge (15.7 ± 1.4 vs. 18.0 ± 3.9 µg/kg; *n* = 6, *P* = 0.57; Fig. 3).

Organ bath experiments showed that pre-incubation with LP44 or cisapride induced a small but statistically significant increase of the contractile response of guinea-pig tracheas to the electrical field stimulation (121.8 ± 5.3%, *n* = 7, *P* = 0.006 and 115.8 ± 5.0%, *n* = 9, *P* = 0.014, respectively; Fig. 4).

In the case of lung parenchyma strips, addition of ACh to the organ bath produced a concentration-dependent

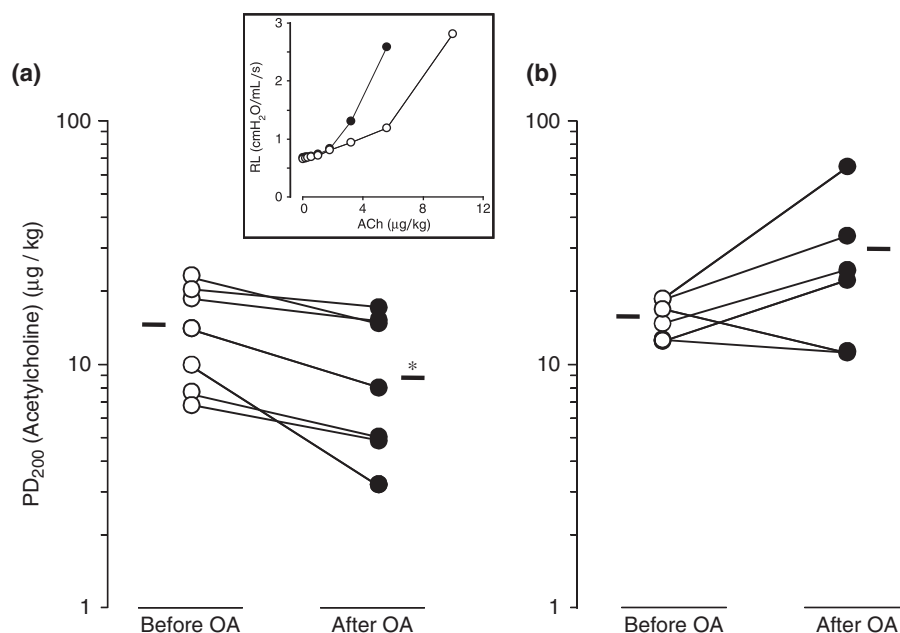


Fig. 1. Airway hyperresponsiveness (AHR) to acetylcholine (ACh) after an antigenic challenge with ovalbumin (OVA), and its abolishment by antiserotonergic drugs. Panel (a) shows that antigenic challenge in guinea-pigs diminished the PD_{200} (dose of ACh that caused a 200% increase of the basal R_L value), i.e. induced AHR. In panel (b), a combination of methiothepin (0.3 mg/kg) plus tropisetron (3.0 mg/kg) completely abolished the antigen-induced AHR to ACh. Horizontal bars correspond to the average. * $P < 0.002$. The inset corresponds to a typical dose-response curve to ACh before (open circles) and after (closed circles) antigenic challenge.

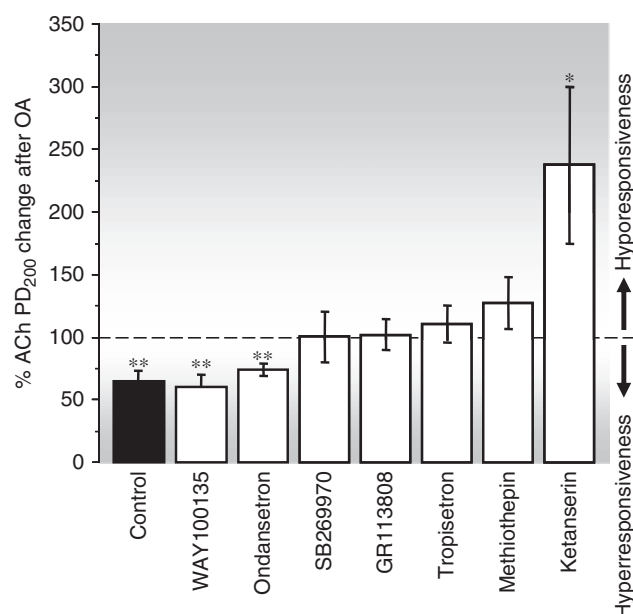


Fig. 2. Effect of different antiserotonergic drugs on the antigen-induced airway hyperresponsiveness (AHR) in guinea-pigs. Bars illustrate the acetylcholine (ACh) 200% provocative dose (PD_{200}) change after antigenic challenge, expressed as percentage of the PD_{200} before challenge. Each group was composed by $n = 5-8$ animals, except the control group ($n = 38$, pooled data). Statistical significance for each group (* $P < 0.05$ and ** $P < 0.002$) was assessed by comparing the percent change of PD_{200} values after antigen challenge with their respective PD_{200} values before challenge using paired Student's t -test. Bars correspond to average, and vertical lines to SEM.

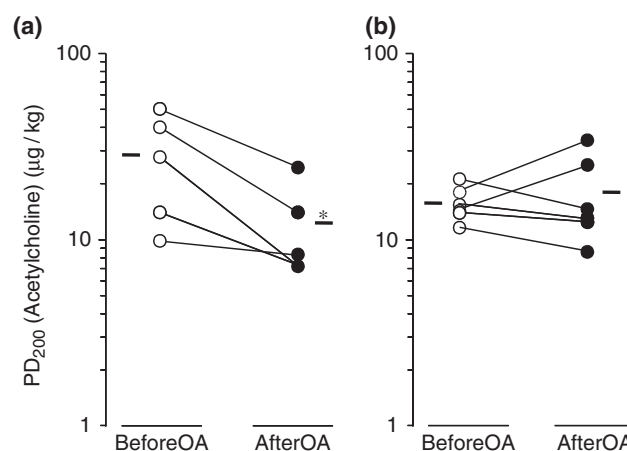


Fig. 3. Effect of bilateral vagal cut in the airway hyperresponsiveness (AHR) to acetylcholine (ACh) after antigenic challenge. Panel (a) shows that the antigenic challenge in guinea-pigs induced AHR to ACh. In panel (b), vagal nerve cut completely abolished the antigen-induced AHR. Horizontal bars correspond to the average. * $P < 0.05$.

contraction of tissues obtained from non-sensitized as well as from sensitized+antigenic challenged guinea-pigs. In non-sensitized strips, neither hexamethonium nor ketanserin modified the EC_{50} and maximum contraction (Fig. 5). By contrast, in sensitized+antigenic challenged tissues, both drugs diminished the EC_{50} and maximum contraction values, but only ketanserin reached statistical significance (Fig. 6).

The 5-HT content in lung homogenates from sensitized guinea-pigs increased approximately ninefold with respect to control animals (Fig. 7). However, when sensitized

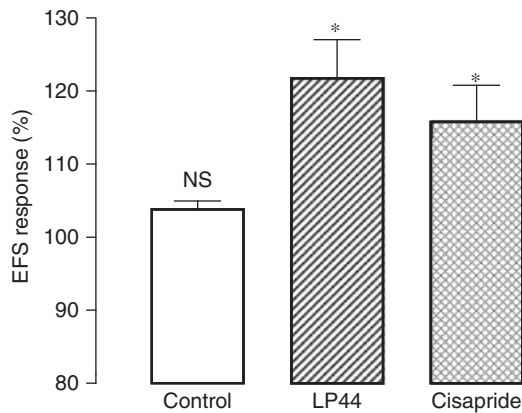


Fig. 4. Effect of LP44 (2.56 μ M; a high-affinity 5-HT₇ receptor agonist) and cisapride (1 μ M, 5-HT₄ agonist) on the contraction induced by electrical field stimulation (EFS; 0.5 Hz, 2 ms and 100 V for 10 s) of the guinea-pig tracheal rings. Bars represent the average, and vertical lines the SEM.

animals were subjected to an antigenic challenge, the 5-HT concentration reverted to values similar to the control guinea-pigs.

Confocal images obtained from non-sensitized guinea-pig lungs showed low fluorescence intensity with antibodies to nicotinic ACh receptor $\alpha 7$ (stained green) and 5-HT (stained red). Tissues from animals sensitized to OVA displayed a stronger fluorescence either for the nicotinic receptor or for 5-HT. Co-localization of the nicotinic receptor and 5-HT, demonstrable by the yellow-orange-punctuated staining in merged images, could only be seen in sensitized tissues (Fig. 8).

Through real-time quantitative PCR, we found that amplification levels of 5-HT_{2A} receptor mRNA in lung tissues or tracheal smooth muscle were not different between control, sensitized and sensitized+antigenic challenged groups (Fig. 9).

Discussion

The results from the present study pointed out that 5-HT_{2A}, 5-HT₄ and 5-HT₇ receptors were involved in

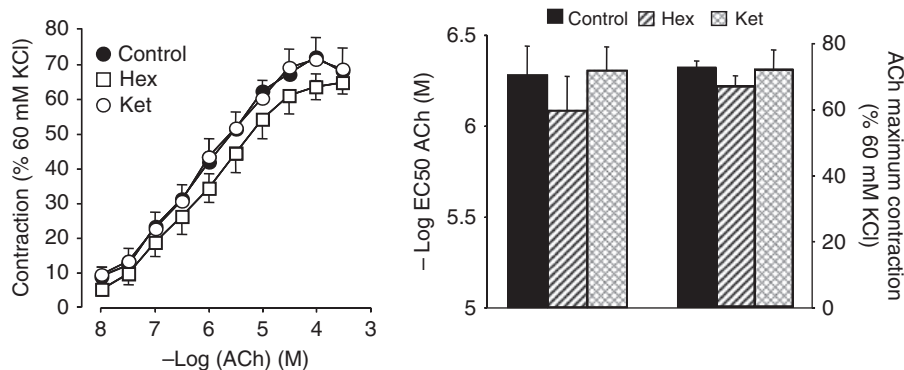


Fig. 5. Effect of hexamethonium (Hex, 30 μ M) and ketanserin (Ket, 10 nM) in the contractile response to acetylcholine (ACh) in lung parenchyma strips from non-sensitized guinea-pigs. Bars represent the average, and vertical lines the SEM.

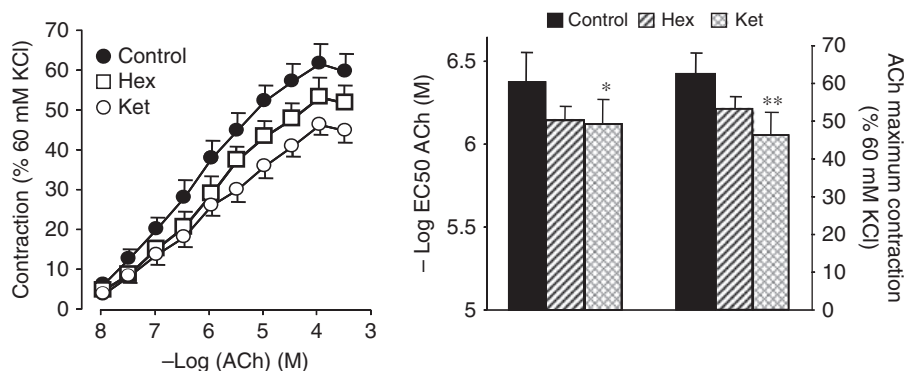


Fig. 6. Effect of hexamethonium (Hex, 30 μ M) and ketanserin (Ket, 10 nM) in the contractile response to acetylcholine (ACh) in lung parenchyma strips from sensitized guinea-pigs. Parenchymal strips from sensitized guinea-pigs were obtained 2 h after an antigenic challenge (ovalbumin, i.v.). * $P < 0.05$ and ** $P < 0.01$ compared with their own control parenchymas.

the development of the AI-AHR to ACh in guinea-pigs. A probable sequence of events is depicted in Fig. 10.

We found that the sensitization procedure *per se* induced an increase in the 5-HT concentration in guinea-pig lung homogenates, and through confocal microscopy, we corroborated that this augmented content was mainly observed in airway epithelium, probably localized to PNECs. It is well known that PNECs can synthesize and release 5-HT [30–33]. Normal PNECs are abundant in the mammalian lung during the perinatal period [34]. Their number decreases rapidly during the first 2 weeks after birth and remains sparse in the healthy adult lung. However, widespread hyperplasia of PNECs is found in

the lungs of adults with some diseases such as chronic bronchitis, emphysema and asthma [31, 35–37]. Interestingly, we found that the nicotinic receptor was co-localized in the same cells in which 5-HT was observed, which is in agreement with a published study performed in hamster lungs [38]. Other research groups have already reported that stimulation of the nicotinic receptor by nicotine induces the release of 5-HT from PNECs [30, 39]. In our study, PNECs might be a potential source of 5-HT, at least in sensitized guinea-pigs, from which 5-HT release may be triggered by ACh during a dose-response curve. Although we do not have direct evidence that ACh releases 5-HT, our pharmacological approach supports this possibility. Thus, a serotonergic component of the

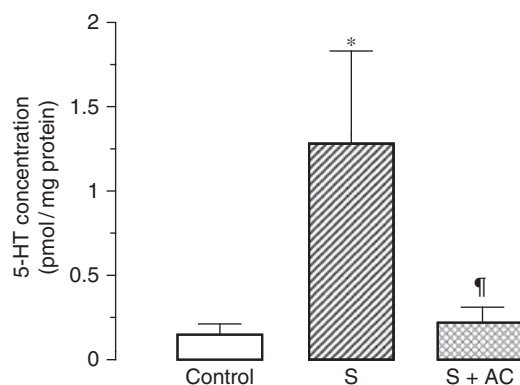


Fig. 7. 5-Hydroxytryptamine (5-HT) concentration in lung parenchyma homogenates from control ($n = 6$), sensitized (S, $n = 5$) and sensitized + antigenic challenged (S+AC, $n = 7$) guinea-pigs. Antigen challenge was accomplished with ovalbumin i.v. 2 h before lung extraction. Bars correspond to average $\pm$ SEM. * $P < 0.05$ compared with the control group. # $P < 0.05$ compared with the sensitized group.

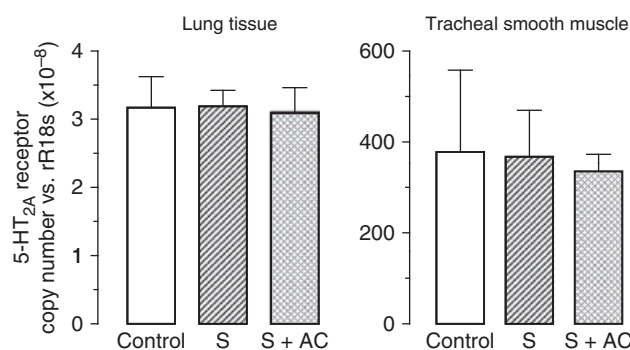


Fig. 9. Quantitative real-time PCR. Levels of mRNA for the 5HT_{2A} receptor normalized to amplified levels of 18S rRNA in lung tissues and tracheal smooth muscle from control, sensitized (S) and sensitized + antigenic-challenged (S+AC) guinea-pigs. Antigen challenge was accomplished with ovalbumin i.v. 2 h before lung extraction. Bars correspond to average $\pm$ SEM of $n = 3$ animals per group. 5-HT, 5-hydroxytryptamine.

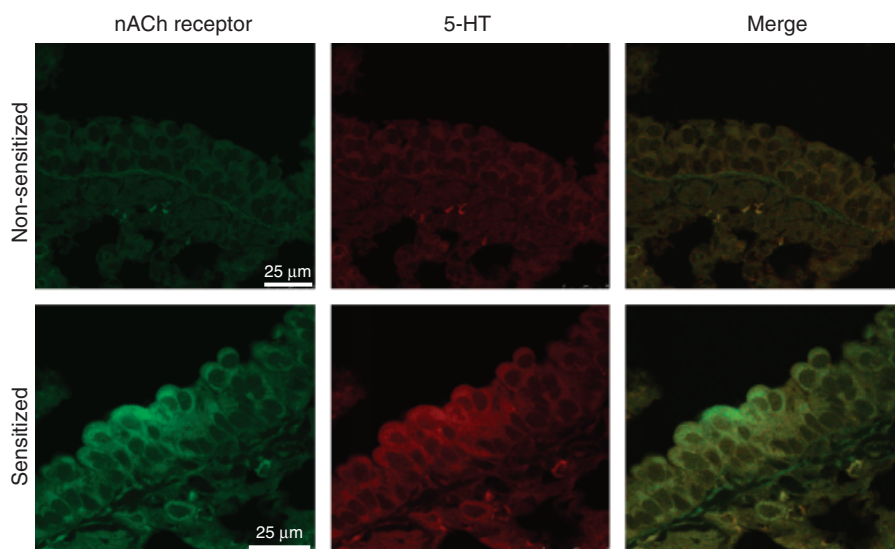


Fig. 8. Confocal microscopy for the nicotinic $\alpha 7$ receptor (nACh receptor) and 5-hydroxytryptamine (5-HT) in guinea-pig airway epithelium. Sensitization to ovalbumin considerably enhanced the immunodetection of both nACh receptor and 5-HT, compared with non-sensitized tissues. Co-localization of nACh receptors and 5-HT could be appreciated by the yellow colouring after merging both images. Bar scales = 25 μ m.

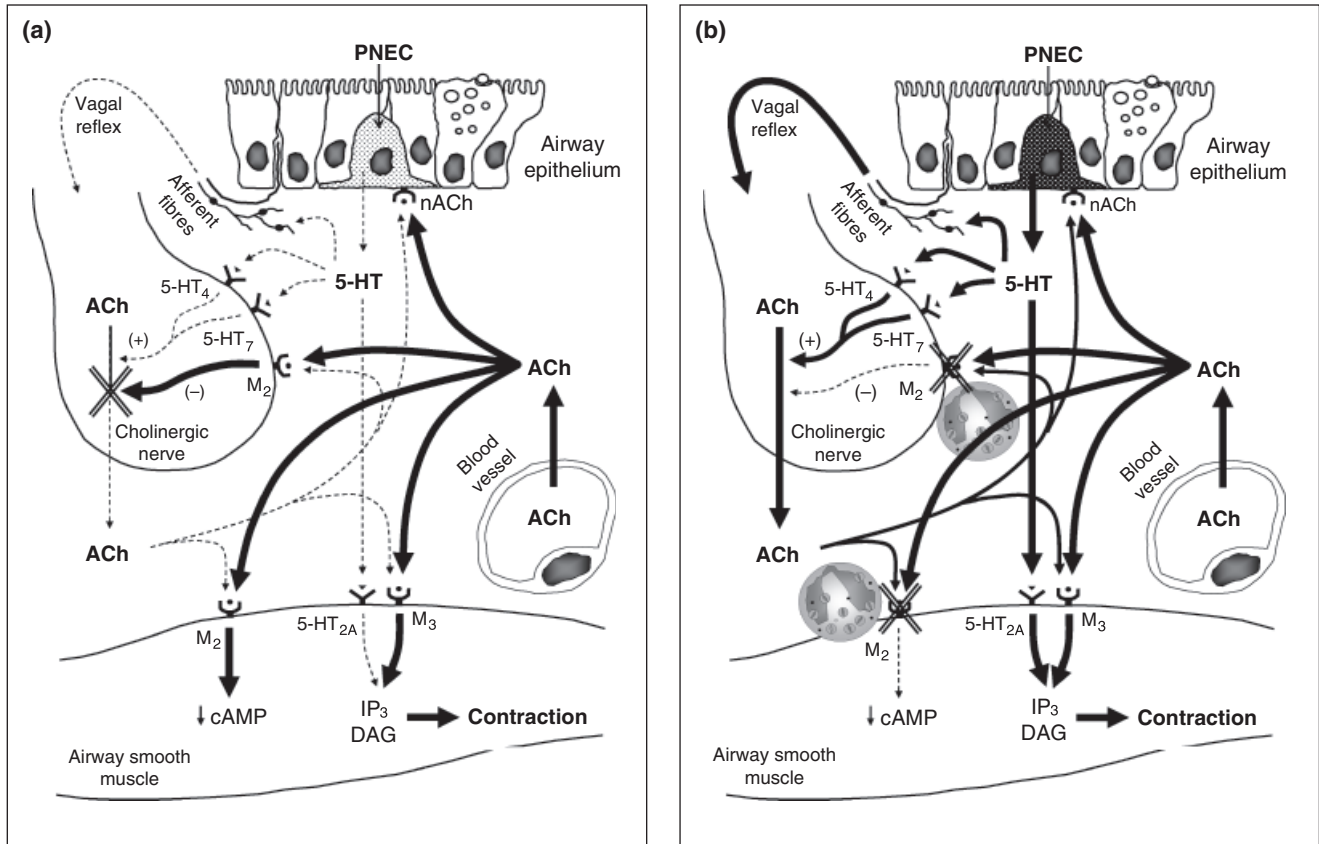


Fig. 10. Events probably occurring during the development of antigen-induced airway hyperresponsiveness to ACh. In non-sensitized guinea-pigs (a), intravenous ACh stimulates muscarinic M_3 receptors to produce airway smooth muscle contraction. Stimulation of M_2 receptors in airway smooth muscle potentiates the contraction through a reduction in the cAMP production. At the pre-junctional level, M_2 stimulation prevents further release of ACh from neuronal ends. Intravenous ACh would also release 5-HT from epithelial neuroendocrine cells. After sensitization, two critical changes might be occurring (b). Firstly, the 5-HT content in pulmonary neuroendocrine cells notably increases, and so its release by ACh is enhanced. 5-HT produces airway smooth muscle contraction through stimulation of 5-HT $_{2A}$ receptors. Secondly, arrival of eosinophils induces deterioration of pre-junctional M_2 , thus leaving unopposed stimulatory mechanisms that promote ACh release, such as those initiated by neuronal 5-HT $_4$ and 5-HT $_7$ receptors. Activation of afferent fibres by 5-HT induces vagal reflex, boosting ACh release. The enhanced ACh release would further potentiate contractile mechanisms. Eosinophils also cause deterioration of M_2 receptors in airway smooth muscle, decreasing the contractile responses to ACh, which now becomes more dependent on 5-HT $_{2A}$ stimulation. ACh, acetylcholine; 5-HT, 5-hydroxytryptamine or serotonin; nACh, nicotinic receptor; IP $_3$, inositol triphosphate; DAG, diacylglycerol; PNEC, pulmonary neuroendocrine cell.

ACh response was introduced in sensitized animals (abolished by ketanserin both *in vivo* and *in vitro*), and this component had a tendency to be reduced by the blockade of nicotinic receptors (hexamethonium in lung parenchyma strips).

A number of studies have demonstrated that airway smooth muscle presents 5-HT $_{2A}$ receptors at the membrane surface [4, 40, 41]. By contrast, the location of pulmonary 5-HT $_4$ and 5-HT $_7$ receptors has been less explored. Through an *in vitro* study of human airways in organ baths, Dupont et al. [42] concluded that 5-HT $_4$ receptors are located at the pre-junctional level of cholinergic nerves, favouring ACh release. In tissues such as the bladder, iris, gastrointestinal and brain, both 5-HT $_4$ and 5-HT $_7$ receptors have also been located at the pre-junctional side of the cholinergic neuronal ends [43–46].

In the present study, we found that activation of either 5-HT $_4$ or 5-HT $_7$ receptors increased the contractile cholinergic responses to electrical field stimulation in guinea-pig tracheas, thus suggesting a possible pre-junctional positioning of these receptors. This is in line with the fact that both 5-HT $_4$ and 5-HT $_7$ receptors are coupled to Gs protein, and thus their activation induces an increment of cAMP, as it is well known that a cAMP increment induces neurotransmitters release in the brain [47] and in airways neural network [48]. Furthermore, Weigand et al. [10] recently reported that activation of pre-junctional 5-HT $_2$ receptors on parasympathetic cholinergic neurons, leading to enhanced ACh release, is required in order to fully develop the antigen-induced contraction of mouse trachea. The enhanced ability of cholinergic nerves to release increasing amounts of ACh has already been postulated by

others as a major mechanism in the development of AHR [49]. This research group has found that sensitization [50], ozone exposure [51] and virus infection [52] enhance the release of ACh through deterioration of pre-junctional M_2 receptors (which, under normal conditions, inhibits ACh release). Specifically, they found that the eosinophil-derived major basic protein allosterically interacts with pre-junctional M_2 receptors, rendering them non-functional. In this context, in our study, the antigenic challenge might exacerbate the eosinophilic inflammatory airway infiltration [53], inducing further deterioration of pre-junctional M_2 receptors. Under these conditions, the second ACh dose-response curve will stimulate the nicotinic receptors of PNECs, causing the release of 5-HT, which in turn will favour an enhanced release of ACh via stimulation of pre-junctional serotonergic receptors in cholinergic ends. In line with these findings, in our *in vivo* experiments, we found that vagal cut and either 5-HT₄ or 5-HT₇ receptor antagonism fully abolished the AI-AHR, thus highlighting the important role of ACh release in this phenomenon.

In *in vivo* animal experiments, 5-HT administration is able to initiate vagal reflexes to produce bronchoconstriction [54, 55]. On the other hand, it is well known that neuroendocrine cells grouped to integrate neuroepithelial bodies are closely related to afferent vagal fibres corresponding to irritant- or C-fibres [56, 57]. Thus, 5-HT released by PNEC after i.v. administration of ACh in sensitized guinea-pigs might be initiating vagal reflexes, which would contribute to the bronchoconstriction but also to further enhance the release of 5-HT. This would explain why vagotomy abolished the AI-AHR to ACh. Interestingly, the results from our *in vitro* experiments mirrored what occurred *in vivo*. Thus, in lung parenchyma strips (in which there is an obvious absence of vagal reflexes), we did not find differences between tissues from non sensitized and sensitized+antigenic challenged guinea-pigs, either in EC50 or maximum responses to ACh (comparisons not shown), but a new 5-HT-dependent component was evident in the sensitized tissues.

It has been well demonstrated that 5-HT_{2A} plays a major role in the acute contraction induced by 5-HT in airway smooth muscle. However, participation of 5-HT_{2A} receptors in AHR has been scarcely investigated. Cazzola et al. [4] demonstrated that 5-HT₂ receptor blockade by ketanserin produced a small but statistically significant decrease of the AHR to methacholine in human asthmatic patients. Likewise, De Bie et al. [9] found that the 5-HT₂ receptor was involved in AHR to methacholine after antigenic challenge in a mouse model of allergic asthma. Our results fully agree with these two previous reports. However, in our study, we found that antagonism of 5-HT_{2A} receptors by ketanserin not only completely blocked the AI-AHR to ACh *in vivo*, but even produced hyporesponsiveness to ACh. Interestingly, ketanserin did not

modify the PD₂₀₀ to ACh in non-sensitized animals nor the EC50 and maximum response to ACh in lung parenchyma strips from non-sensitized animals, suggesting that the above-described hypo-reactive effect of ketanserin was not occurring or was negligible if sensitization was lacking. Thus, it seems that antigenic challenge not only added a 5-HT_{2A} receptor-dependent component to the ACh response, but also deteriorated the expected normal contractile response to ACh. A possible explanation for this last speculation might be related to M_2 receptor blockade. Thus, as we commented above, it has already been demonstrated that pre-junctional M_2 receptors are deteriorated by major basic protein, which is released in large amounts during an antigenic challenge [51]. However, it is well known that M_2 receptors are also located in the airway smooth muscle, where they can open unspecific cation channels and inhibit cAMP production, both actions favouring contraction [58, 59]. Thus, it is reasonable to speculate that major basic protein might also be allosterically interacting with these post-junctional M_2 receptors, rendering them non-functional, in the same way as it does with pre-junctional M_2 receptors. This would theoretically reduce the capability of ACh to induce airway smooth muscle contraction, which then would become largely dependent on 5-HT contribution through activation of 5-HT_{2A} receptors in airway smooth muscle. This hypothesis warrants further investigation.

We discarded that the increased dependence of ACh response on 5-HT was due to up-regulation of the 5-HT_{2A} receptors, inasmuch as mRNA was not different among control, sensitized and sensitized+antigenic challenged lung tissues.

According to our results, we found that 5-HT was involved in the generation of AI-AHR to ACh in guinea-pigs. 5-HT_{2A}, 5-HT₄ and 5-HT₇ receptors seem to be particularly involved in this phenomenon. Participation of 5-HT might probably be favoured by the enhancement of the PNECs 5-HT content observed after sensitization.

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