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Dysfunctional muscarinic M_2 autoreceptors in vagally induced bronchoconstriction of conscious guinea pigs after the early allergic reaction

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Abstract

We studied the function of autoinhibitory muscarinic M_2 receptors on vagal nerve endings in the airways of conscious, unrestrained, ovalbumin-sensitized guinea pigs after the early and late allergic reaction. For this purpose, the effects of the selective muscarinic M_2 receptor antagonist gallamine were examined on unilateral vagus nerve stimulation-induced bronchoconstriction, which was determined as an increase in basal respiration amplitude, measured as changes in pleural pressure. Under control conditions, i.e., before antigen challenge, a significant increase in the pleural pressure was found after inhalation of 0.1 mM and, even more pronounced, 1.0 mM gallamine, at medium stimulation frequencies (2–16 Hz), leading to a leftward shift of the frequency-response curve. After inhalation of 10 mM of gallamine, a complete reversal of the left-shift was observed and the frequency-response curve was depressed. However, 6 h after challenge with ovalbumin (i.e., after the early allergic reaction) no increase in nerve stimulation-induced bronchoconstriction by gallamine was found; a decrease in this bronchoconstriction was again observed with the highest concentration. At this moment, bronchial responsiveness to histamine was enhanced 4.5-fold compared to control, i.e., prior to antigen provocation. Both after the late allergic response (24 h after challenge; 1.6-fold histamine hyperresponsiveness) and 4 days after allergen challenge (normal histamine responsiveness) the gallamine-induced potentiation of the bronchoconstriction was restored, similar to the responses under control conditions. The results clearly demonstrate that prejunctional muscarinic M_2 receptors control bronchoconstriction in conscious, unrestrained guinea pigs in vivo. Furthermore, these autoinhibitory receptors appear to be completely dysfunctional after the early allergic phase, but their function is largely restored after the late phase. The results indicate that dysfunction of autoinhibitory muscarinic M_2 receptors might contribute to the strongly enhanced responsiveness to histamine after the early allergic response.

Keywords: (Conscious, unrestrained guinea pig); Vagus nerve stimulation; Muscarinic M_2 receptor, prejunctional; Receptor dysfunction; Muscarinic receptor antagonist; Allergic reaction, early, late; Bronchial hyperresponsiveness

1. Introduction

In the airways, the release of acetylcholine from vagus nerve endings is under the inhibitory control of muscarinic autoreceptors (Fryer and Maclagan, 1984) of the M_2 subtype, while postjunctional muscarinic M_3 receptors are involved in airway smooth muscle contraction (Barnes et al., 1988; Ten Berge et al., 1993). These prejunctional muscarinic M_2 receptors have been demonstrated in many species including guinea pig (Fryer and Maclagan, 1984; Faulkner et al., 1986) and man (Minette and Barnes, 1988), and they have been suggested to become dysfunctional in asthmatics (Ayala and Ahmed, 1989; Minette et al., 1989) and in guinea pigs after acute viral infection (Fryer and Jacoby, 1991) and ozone exposure (Schultheis et al., 1994) and after antigen challenge of sensitized animals (Fryer and Wills-Karp, 1991; Ten Berge et al., 1995). Such muscarinic M_2 receptor dysfunction may partly explain the increased bronchial responsiveness which is one of the most important features of asthma in humans (Cockcroft et al., 1977) and in models of asthma in experimental animals (Wanner et al., 1990), since it has been shown that part of the bronchoconstrictor response to histamine in guinea pigs in vivo is mediated via the vagus nerve (Hulbert et al., 1985; Santing et al., 1995b), and that

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the muscarinic receptor antagonist ipratropium bromide attenuates histamine-induced bronchoconstriction in humans (Ayala and Ahmed, 1989).

In a previous study, the function of inhibitory muscarinic M_2 receptors was examined on electrical field stimulation-evoked twitch contractions of guinea pig isolated tracheal preparations using selective muscarinic M_2 receptor antagonists (Ten Berge et al., 1995). With this method we were able to demonstrate that muscarinic M_2 receptor function was strongly impaired after the early allergic reaction and restored after the late response; however, information on muscarinic M_2 receptor function in vivo could not be provided. In the above-mentioned studies performed by Fryer and colleagues, guinea pigs were anaesthetized and both vagus nerves were cut in order to stimulate the distal portions electrically. Since the experiments were terminal, animals were only used once, excluding an intra-animal comparison of data. We therefore developed a model that enabled us to measure vagus nerve stimulation-induced bronchoconstriction repeatedly in conscious, unrestrained guinea pigs, by implanting a bipolar stimulation electrode around the intact vagus nerve. Bronchoconstriction was observed as an increase in respiration amplitude, measured as pleural pressure with a small intrapleural balloon (Santing et al., 1992). In order to determine the function of the prejunctional muscarinic M_2 receptors in our guinea pig model of allergic asthma (Santing et al., 1994a), the effects of gallamine on vagally induced bronchoconstriction were examined 4 days before ovalbumin challenge, after the early (6 h) or late allergic reaction (24 h after antigen) and 4 days after the ovalbumin challenge. Furthermore, prior to all gallamine experiments, bronchial responsiveness to aerolised histamine was assessed.

2. Materials and methods

2.1. Animals

Outbred guinea pigs of either sex were sensitized to ovalbumin at 3 weeks of age, when they weighed approximately 300 g. A shift to heat-labile immunoglobulin E class antibodies (as determined by passive cutaneous anaphylaxis) was obtained by adding aluminium hydroxide in a final concentration of 100 mg/ml to a solution of 100 μg ovalbumin per ml saline. The antigen solution was injected intraperitoneally (0.5 ml) and intradermally in the proximity of seven lymph nodes in the paws, lumbar regions and neck (70 μl each). This procedure is a modification of the method of Andersson (1980), as described by Van Amsterdam et al. (1989). The animals were operated in week 3 following sensitization and were used in weeks 4–8, when they weighed 500–800 g.

All protocols described were approved by the University of Groningen Animal Health Committee, which is responsible for assuring the care and proper use of experimental animals.

2.2. Surgery and measurement of airway function

Animals were anaesthetized with a combination of halothane, N_2O and O_2, and the head, dorsal and ventral surface of the neck and the right side of the chest were shaved and subsequently scrubbed with chlorhexidine solution (1.0%). An incision was made on the head, and skull membranes in the vicinity of the bregma were removed; three small screws were inserted into the skull. Two stainless-steel electrodes, bent into a circle (inner diameter 0.15 cm) with a slight opening, were each soldered to 10 cm of flexible, isolated electrode wire (Biomed Wire, Cooner Wire Company, Los Angeles, CA, USA) and fixed at 2 mm distance by the use of dental acrylic glue. At the other end of the wires a female microplug, made of an IC foot, was soldered. The circular electrodes were embedded in a small piece of longitudinally opened silicone tubing (i.d. 0.64 mm, o.d. 1.19 mm; Silastic, Dow Corning, Ann Arbor, MI, USA) to provide isolation from surrounding tissue. At the right side of the trachea, the vagus nerve was found and cautiously exposed using an operating microscope. Then, the bipolar electrode and the silicone tubing were carefully placed around the vagus nerve after which the tubing was closed at both ends, using 7-0 silk suture (Ethicon, Norderstedt, Germany). The electrode was subsequently anchored to the connective tissue covering the trachea, using one stitch of 7-0 silk suture. The electrode wire was run subcutaneously to emerge at the crown of the head of the guinea pig, where the microplug was fixed between the three screws with dental acrylic glue.

Airway function was assessed by measuring pleural pressure (P_{pl}) as described by Santing et al. (1992), using a small latex balloon connected to a saline-filled piece of tubing surgically implanted inside the thoracic cavity. The cannula was passed subcutaneously and permanently attached to the neck of the animal. After connection via a fluid-filled cannula to a pressure transducer (Gould P23ID, Gould Medical, Bilthoven, Netherlands), P_{pl} was measured in cmH_2O using an on-line computer system. The animals were permitted to recover from the operation until preoperative body weight was restored (usually 10–12 days). Over the time period of experimentation (1–5 weeks after surgery) no visual sign of inflammation was observed at the sites of surgery, and baseline P_{pl} values remained stable during repeated measurements. Changes in P_{pl} are linearly related to changes in airway resistance (Santing et al., 1992) and hence can be used as a sensitive index for histamine-, allergen- and vagus nerve stimulation-induced bronchoconstriction.

2.3. Provocation techniques and procedures

On the first day of the experimental protocol, the animals were placed unrestrained inside a specially de-

signed animal provocation cage (Santing et al., 1992), but were not connected to the pressure transducer. The animals were exposed to three saline aerosols for 3 min each, separated by 10-min intervals. Aerosols were produced by a DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA). On day 2 this protocol was repeated, with the animals connected to the measuring system. From day 3 onward, animals were challenged with histamine, ovalbumin, gallamine or ipratropium aerosols, each time preceded by two saline challenges as described above.

Histamine provocation started with a 25 μg/ml solution of histamine in saline, followed by increasing dosage steps of 25 μg/ml. Challenges lasted 3 min each and were separated by 10-min intervals. Animals were challenged until the pleural pressure increased by more than 100% for at least 3 min. The concentration of histamine giving a 100% increase in pleural pressure (PC_{100}) was calculated by intrapolation. To study bronchial responsiveness, histamine inhalation was started at 5:00 h or at 23:00 h after ovalbumin challenge, i.e., after the early or late phase, respectively.

Single allergen provocations were performed with a concentration of 0.1% (g/v; four animals) or, if neccessary, 0.3% ovalbumin in saline (six animals), according to Santing et al. (1994a). The aerosol challenge was continued until a 2-fold increase in pleural pressure was measured, to a maximum of 3 min.

2.4. Vagus nerve stimulation

Stimulation of the vagus nerve, applied via a home-made constant current biphasic pulse generator, resulted in an increase in pleural pressure, which was registered on a flatbed recorder. Stimulation periods were 10 s to obtain a plateau increase in the pleural pressure for at least 5 s; pulse duration was 0.1 ms. Prior to each experiment, the current intensity was chosen so as to obtain a 1.5- to 1.7-fold enhancement of the pleural pressure at 8 Hz, and was kept constant throughout that particular experiment. Stimulation-response curves were made with continuously doubling frequencies (1–2–4–8–16–32 Hz). All stimulations were performed twice, separated by a short interval during which respiration amplitude returned to basal levels for at least 10 s; average values obtained at each frequency were used.

2.5. Experimental protocol

Bronchial responsiveness to histamine was determined on 2 consecutive days; the second PC_{100} value was taken as control. Thirty minutes after the last histamine challenge of the second day, when pleural pressure had returned to baseline, a saline aerosol (3 min) was given to the guinea pigs; a nerve stimulation-response curve was made 15 min later. Next, to study the function of prejunctional inhibitory muscarinic M_2 receptors, an aerosol containing the selective muscarinic M_2 receptor antagonist gallamine (0.1 mM in saline) was given for 3 min, and after 15 min a stimulation-response curve was recorded. Further nerve stimulation-response curves were similarly obtained after inhalation of higher gallamine concentrations (1.0 and 10 mM in saline). In preliminary studies no effects on stimulation-induced bronchoconstriction were found after inhalation of 1 μM and 10 μM gallamine in saline (data not shown).

Four days later these guinea pigs (group 1) were challenged with ovalbumin, and bronchial responsiveness to histamine was determined, starting 5 h after provocation. Thereafter, i.e., at 6 h after allergen challenge, nerve stimulation-response curves were made after saline and 0.1, 1.0 and 10 mM gallamine, as described above.

Four days after this experiment, histamine responsiveness was re-examined and gallamine effects were studied on stimulation-induced bronchoconstriction again. Finally, again 4 days later, the effect of the non-selective muscarinic antagonist ipratropium bromide (1.0 mM in saline) on nerve stimulation-induced bronchoconstriction was investigated using the same protocol as described for gallamine.

In a second group of guinea pigs (group 2) bronchial responsiveness to histamine and effects of gallamine on the evoked bronchoconstriction were determined under control conditions, as described above. Four days later the animals were challenged with ovalbumin, and histamine responsiveness was measured after the late allergic reaction, i.e., 23 h after provocation. Thereafter, i.e., at 24 h after antigen challenge, nerve stimulation-response curves were made after exposure to saline and 0.1, 1.0 and 10 mM gallamine, as described above.

2.6. Drugs

The following substances were used: gallamine triethiodide, histamine dihydrochloride, ovalbumin (grade III)

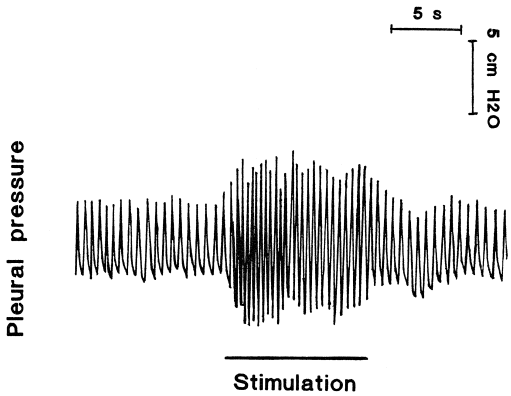

Fig. 1. Typical example of the effect of vagus nerve stimulation (8 Hz, 0.1 ms, 10 s, 7.5 mA) on the pleural pressure in the airways of conscious, unrestrained guinea pigs. The amplitude represents the differences between inspiration (upper level) and expiration (lower level).

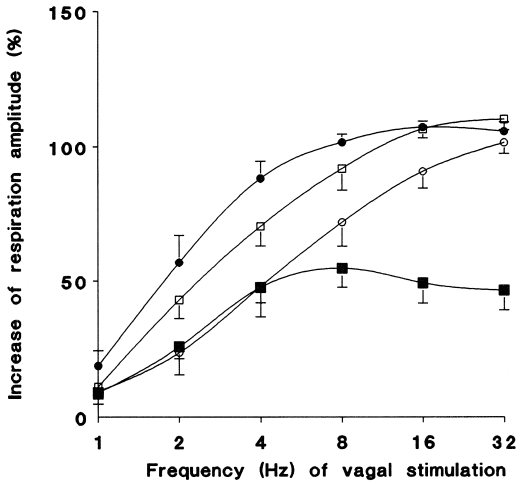

Fig. 2. Effect of the M_2-selective muscarinic receptor antagonist gallamine on vagally induced bronchoconstriction in IgE-sensitized guinea pigs. Gallamine was given for 3 min, 15 min prior to nerve stimulation, as aerosol containing 0.1 mM (□), 1.0 mM (●) or 10 mM (■) of the drug; saline aerosol was used as control (○). Results are expressed as means ± S.E.M. of stimulation-induced increases in respiration amplitude of 6 experiments (each frequency applied in duplicate).

(Sigma, St. Louis, MO, USA) and aluminium hydroxide (Janssen Chimica, Beerse, Belgium). Ipratropium bromide was a gift from Boehringer Ingelheim (Ingelheim am Rhein, Germany).

2.7. Data analysis

Bronchial hyperresponsiveness to histamine is expressed as the ratio of PC_{100} values prior to and after ovalbumin provocation (Santing et al., 1994a). Effects of nerve stimulation, at all frequencies applied, are expressed as stimulated/basal respiration amplitude. Results are expressed as means ± S.E.M. of n determinations. Statistical significance was assessed by paired Student's t-test and P values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Airway responses

Guinea pigs that received ovalbumin between 4 and 8 weeks after sensitization developed an early allergic reaction that started with a sharp increase in pleural pressure which was maximal within 15 min and lasted for 2–5 h. The late response started at about 8 h after allergen provocation and ended within 23 h of the challenge (Santing et al., 1994a).

Baseline pleural pressure before the first histamine challenge was 5.39 ± 0.42 cmH_2O ($n = 10$). The mean dose of

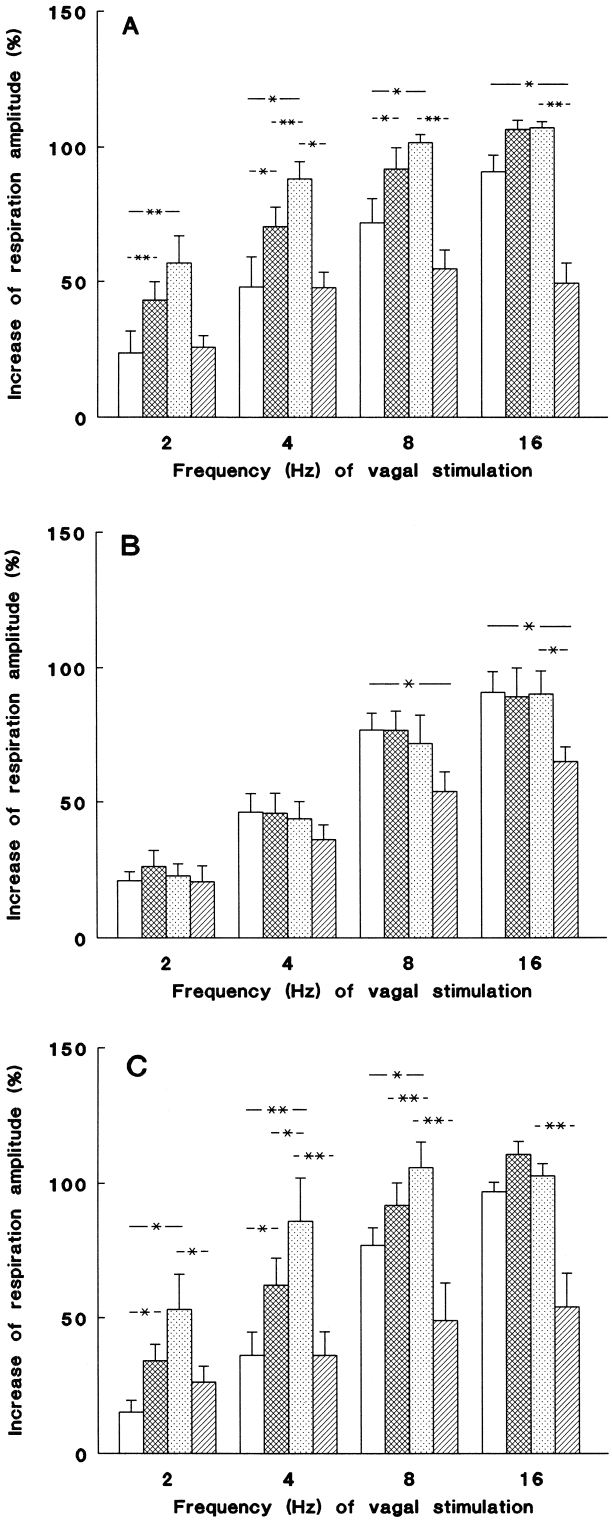

Fig. 3. Effect of the M_2-selective muscarinic receptor antagonist gallamine on vagally induced bronchoconstriction in IgE-sensitized guinea pigs (group 1); A: control (before allergen challenge); B: after the early allergic reaction; C: at least 4 days after allergen challenge. Gallamine was given for 3 min, 15 min prior to nerve stimulation, as aerosol containing 0.1 mM (cross-hatched bars), 1.0 mM (dotted bars) or 10 mM (hatched bars) of the drug. Saline aerosol was used as control (open bars). Results are expressed as means ± S.E.M. of stimulation-induced increases in respiration amplitude of 6 experiments (each frequency applied in duplicate); significant differences are indicated by * $P < 0.05$; ** $P < 0.01$.

histamine required to double this pleural pressure, used as a measure of bronchial responsiveness, in control determinations was 76.9 ± 9.1 μg/ml ($n = 10$). Six hours after allergen challenge bronchial responsiveness was increased 4.5 ± 0.8-fold ($n = 6$; $P < 0.01$); after the late reaction responsiveness was increased 1.6 ± 0.1-fold ($n = 4$; $P < 0.05$). Four days after antigen, histamine concentrations required to double pleural pressure had returned to normal (79.6 ± 13.6 μg/ml; $n = 6$).

Stimulation of the right vagus nerve resulted in a rapid and frequency-dependent increase in the respiration amplitude (Figs. 1 and 2). This increase started within 1 s, usually reached a maximal value within 5 s, and decreased to baseline within 5 s after stimulation was stopped. Furthermore, this increase appeared to level off at a stimulation frequency of 32 Hz, reaching a maximum of approximately 200% of control. The optimal current intensities determined just before the start of the first experiment slightly varied between the animals, yielding an average value of 7.8 ± 0.9 mA ($n = 10$). This value was not significantly altered in the subsequent experiments (i.e., at 6 h or 24 h or 4–5 days after antigen challenge) in these animals. In addition to the increase in the amplitude, the respiration frequency also changed during nerve stimulation; however, these changes showed some variability both between different experiments with one animal and between different animals. Therefore, only the respiration amplitude was used as an index for bronchoconstriction.

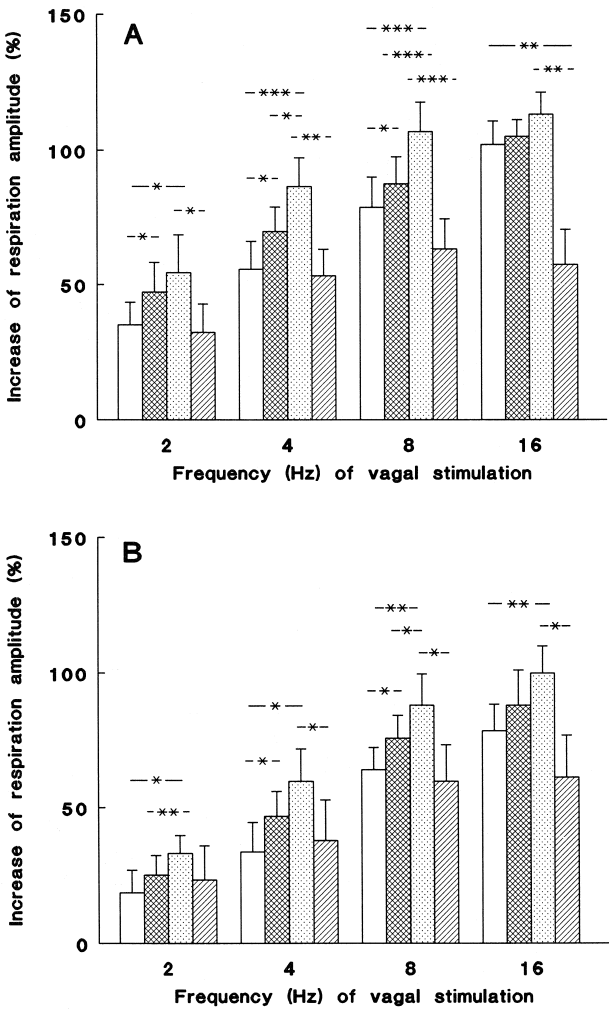

Fig. 4. Effect of the M_2-selective muscarinic receptor antagonist gallamine on vagally induced bronchoconstriction in IgE-sensitized guinea pigs (group 2); A: control (before allergen challenge); B: after the late allergic reaction. Gallamine was given for 3 min, 15 min prior to nerve stimulation, as aerosol containing 0.1 mM (cross-hatched bars), 1.0 mM (dotted bars) or 10 mM (hatched bars) of the drug. Saline aerosol was used as control (open bars). Results are expressed as means ± S.E.M. of stimulation-induced increases in respiration amplitude of 4 experiments (each frequency applied in duplicate); significant differences are indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

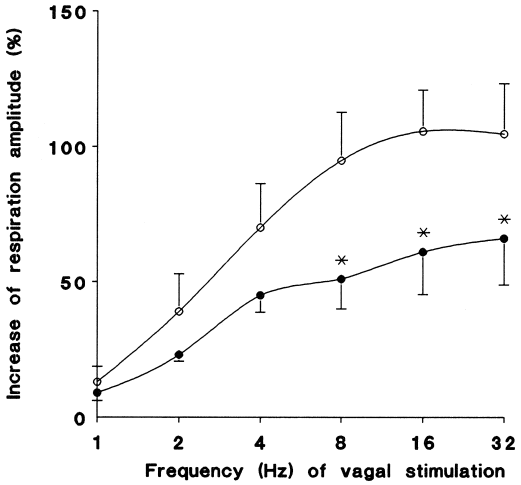

Fig. 5. Effect of the non-selective muscarinic antagonist ipratropium on vagally induced bronchoconstriction in IgE-sensitized guinea pigs (group 1). Ipratropium was given for 3 min, 15 min prior to nerve stimulation, as aerosol containing 1.0 mM (●) of the drug; saline aerosol was used as control (○). Results are expressed as means ± S.E.M. of stimulation-induced increases in respiration amplitude of 5 experiments (each frequency applied in duplicate); * $P < 0.05$ compared to control.

3.2. Effect of muscarinic antagonists on nerve stimulation-induced bronchoconstriction

Before allergen challenge, guinea pigs (groups 1 and 2) showed a potentiation of the increase in the respiration amplitude at all frequencies of stimulation (left-shift of the frequency-response curve) after inhalation of gallamine 0.1 mM (Fig. 2). A further left-shift was observed with gallamine 1.0 mM; however, after inhalation of 10 mM gallamine, a complete reversal of the left-shift was observed and the frequency-response curve was depressed (Fig. 2, Fig. 3A and Fig. 4A).

Six hours after ovalbumin provocation (i.e., after the early allergic reaction) no increase in the stimulated respiration amplitude was observed with 0.1 mM and with 1.0 mM gallamine, while a decrease in the response was found with 10 mM gallamine (Fig. 3B). Four days later, the potentiation of nerve stimulation-induced bronchoconstric-

tion with 0.1 and 1.0 mM gallamine was restored, and a decrease was found with the highest gallamine concentration (Fig. 3C), very similar to the responses obtained before antigen challenge.

The function of muscarinic M_2 receptors on vagal nerve endings in the airways after the late allergic reaction was studied in the second group of animals. Gallamine (0.1 and 1.0 mM) potentiated the vagally induced bronchoconstriction in a dose-dependent manner; again a decrease in bronchoconstriction was observed with 10 mM gallamine (Fig. 4B).

With 1 mM ipratropium, given by inhalation to the first group of guinea pigs 4 days after the last gallamine experiment, a 40–50% decrease in the stimulated respiration amplitude (statistically significant at 8, 16 and 32 Hz) was observed (Fig. 5); a 10-fold higher dose did not produce a greater effect (not shown).

4. Discussion

The present study was designed to establish the function of prejunctional inhibitory muscarinic M_2 receptors in the airways of conscious, unrestrained guinea pigs both after the early and after the late allergic reaction. To this end, a model was developed in which bronchoconstriction was evoked in conscious, unrestrained guinea pigs, by stimulation of a vagus nerve, and the effects of the selective muscarinic M_2 receptor antagonist gallamine on vagus nerve stimulation-induced bronchoconstriction were examined. The model was developed in an attempt to study airway function under unanaesthetized conditions, in order to prevent any influence of anaesthetics on airway function (cf., Vettermann et al., 1989; Skornik and Brain, 1990), and to use intact vagus nerves, in contrast to earlier work on allergen-induced muscarinic M_2 receptor dysfunction in guinea pig airways in vivo (Fryer and Wills-Karp, 1991).

4.1. Vagus nerve stimulation

In the present experiments, stimulation of the right vagus nerve was performed at frequencies ranging from 1 to 32 Hz, i.e., a range comprising both those medium frequencies that pass the ganglia without filtering as well as those high and low frequencies that are indeed effectively filtered (Myers and Undem, 1991). It was observed that bronchoconstriction hardly increased from 16 to 32 Hz, probably as a result of this ganglionic filtering (Myers and Undem, 1991), and that the highest frequency applied (32 Hz) induced a 2-fold increase in respiration amplitude. These results are in agreement with the finding that maximal unilateral stimulation of the vagus nerve in cats induces a 2-fold increase in pulmonary resistance (Olsen et al., 1965). By contrast, much larger (5- to 10-fold) increases in pulmonary resistance may be obtained by bilateral vagus nerve stimulation (Olsen et al., 1965; cf., Fryer and Wills-Karp, 1991), but this was regarded unethical in our conscious and spontaneously breathing animals.

4.2. Muscarinic receptor function

Muscarinic autoreceptors of the M_2 subtype provide negative feedback control on neurotransmitter release from vagus nerve endings in the airways (Barnes et al., 1988). In the present study, the function of these receptors was investigated after the early and after the late allergic reaction, using the muscarinic receptor antagonist gallamine, which was chosen because of its high functional M_2/M_3 selectivity (Ten Berge et al., 1993). In order to avoid any influence of residual gallamine given after the early reaction, separate animals were used to study the effect of the late reaction on the muscarinic M_2 receptor function.

Under control conditions, i.e., prior to antigen challenge, a significant potentiation of vagally induced bronchoconstriction was found with 0.1 mM and, even more pronounced, 1.0 mM gallamine, given by aerosol, at medium stimulation frequencies (from 2 to 16 Hz). With 10 mM of this antagonist, no left-shift of the frequency-response curve occurred and a clear (50%) depression of the vagally induced bronchoconstriction was obtained at 8 Hz and higher stimulation frequencies. These results indicate that after inhalation of 0.1 and 1.0 mM gallamine muscarinic M_2 receptors located prejunctionally were selectively blocked, resulting in loss of its autoinhibitory function during vagal stimulation, and that with 10 mM gallamine postjunctional muscarinic M_3 receptors were also blocked. The observation that the non-selective muscarinic receptor antagonist ipratropium bromide, inhaled in a maximally effective concentration (Santing et al., 1995b), similarly decreased nerve-induced bronchoconstriction by approximately 50% may indicate that part of the vagally induced bronchoconstriction in our experiments is of non-cholinergic, probably excitatory non-adrenergic non-cholinergic (NANC), origin.

Recently, Boot and Bond (1992), who stimulated the right vagal nerve of anaesthetized guinea pigs with pulses of 1 ms duration, found that atropine (1 mg/kg i.v.) reduced the bronchoconstriction by 54% only, the remaining part being almost abolished following depletion of neurokinins by capsaicin pretreatment.

Nevertheless, our results were unexpected, for two reasons. First, we used a pulse duration of 0.1 ms, because in guinea pig right vagus nerve-main bronchus preparations such short pulses are found to induce submaximal (80%) cholinergic twitch contractions but only very minor secondary excitatory NANC responses (Undem et al., 1990). Second, Fryer and Wills-Karp (1991) have described that atropine (1 mg/kg i.v.) abolishes the vagally induced bronchoconstriction of anaesthetized guinea pigs. In their study both vagal nerves were cut and stimulation of the distal portions was performed using 0.2 ms pulses. Perhaps

our findings indicate that in the freely moving, normally breathing guinea pig, even short (0.1 ms pulses) stimulation of the right vagus nerve may activate some neurokinin release from excitatory NANC nerves, which could be of physiological relevance.

After the early allergic reaction, at 6 h after challenge with ovalbumin, no increase in nerve stimulation-induced bronchoconstriction was found with gallamine, indicating a complete loss of prejunctional muscarinic M_2 receptor function. A decrease in this bronchoconstriction was however still observed with the highest concentration of gallamine, which demonstrates that postjunctional muscarinic M_3 receptors are not influenced at all. This is in agreement with previous studies on muscarinic receptor function in allergen-challenged animals, which unanimously indicated unaltered postjunctional sensitivity, even when prejunctional dysfunction was present, in vivo (Fryer and Wills-Karp, 1991) or in vitro (Larsen et al., 1994; Ten Berge et al., 1995). Twenty-four hours after allergen provocation in a separate group of guinea pigs, a clear gallamine-induced increase in bronchoconstriction was again observed with 0.1 and 1.0 mM, indicating that muscarinic M_2 receptor function was largely restored at this point of time, while with 10 mM gallamine the evoked increase in pleural pressure was suppressed as usual. As expected from this observation, muscarinic M_2 receptor function examined 4 days after ovalbumin challenge in the first group was also completely restored and similar to that of the control. These data agree with the results obtained in an earlier ex vivo study (Ten Berge et al., 1995), in which it was demonstrated, using four different muscarinic M_2 receptor antagonists, that muscarinic M_2 receptor function was significantly decreased in isolated tracheal preparations from guinea pigs killed after the early allergic reaction, whereas this function was largely restored at 24 h, i.e., after the late response.

Fryer and Wills-Karp (1991), using bilateral vagus nerve stimulation of guinea pigs sensitized with ovalbumin (10 mg/kg i.p. on days 1, 3, 5) and subsequently exposed to five aerosols of 5% ovalbumin for 5 min/day on days 20–24, found a marked prejunctional muscarinic M_2 receptor dysfunction at 24 h after the last antigen provocation. These findings would suggest that after repeated antigen exposure, compared to a single challenge as performed in the present study, the receptor dysfunction becomes more persistent. However, our previous ex vivo study has shown that also with repeated (once daily for 4 consecutive days) antigen provocation, the strong prejunctional muscarinic M_2 receptor dysfunction, as observed in the trachea at 6 h, was restored at 24 h after the last antigen challenge to a similar extent as found with single antigen provocation (Ten Berge et al., 1995).

It should be mentioned, however, that the procedures both for the sensitization and the challenges were different; moreover, the amounts of antigen administered by Fryer and Wills-Karp (1991) were much higher. This may have resulted in a different profile of inflammation in the airways. Comparison of the results for bronchoalveolar lavage performed by both laboratories at 24 or 27 h after the last antigen provocation showed remarkable differences indeed: a marked increase in lymphocytes (2.7-fold) and macrophages (2.3-fold) was found by Fryer and Wills-Karp (1991) whereas no significantly increased pulmonary influx of these cells was found in our laboratory (Santing et al., 1994b). As discussed by Fryer and Wills-Karp, cleavage of sialic acid residues from the muscarinic M_2 receptor by lymphocyte- and macrophage-derived neuroaminidase may be involved in the diminished high-affinity binding of acetylcholine to the receptor.

We found a higher increase in the influx of eosinophils (5.1-fold) and notably of neutrophils (17.3-fold) in the lumen of the lungs than did Fryer and Wills-Karp (3.1-fold and 4.7-fold, respectively). Since eosinophil-derived polycationic proteins, in particular major basic protein, may act as allosteric antagonists of muscarinic M_2 (not of M_3) receptors (see Fryer and Jacoby, 1993), and newly infiltrated eosinophils have the highest activation state during the early allergic reaction (Santing et al., 1995a), it might be envisaged that the muscarinic M_2 receptor dysfunction observed by us at 6 h after allergen inhalation is driven by eosinophils to an important extent.

It should be noted that at 6 h after antigen challenge, when prejunctional muscarinic M_2 receptors appear to be dysfunctional, the control stimulation-response curve in the absence of gallamine was not different from that obtained prior to antigen challenge, when muscarinic M_2 receptors were functioning normally. Vagal sensitivity was unaltered, as judged from the similar optimal current intensities established at these time points. Although we have currently no sound explanation for this finding, it agrees with the experiments of Fryer and Wills-Karp (1991) in which the treshold voltage at 2 Hz was not different between control and antigen-challenged guinea pigs, though at 5 and 15 Hz some increase in vagally induced bronchoconstriction was found in the antigen-challenged animals compared with controls, which was significant at 15 Hz.

4.3. Muscarinic receptor dysfunction and in vivo bronchial reactivity

Bronchial responsiveness to histamine in vivo was also evaluated in the present study and found to be enhanced to a higher extent after the early (4.5-fold) than after the late allergic response (1.6-fold), in agreement with Santing et al. (1994a) and Ten Berge et al. (1995). Four days after allergen provocation histamine responsiveness had returned to normal. The more pronounced histamine hyperresponsiveness after the early than after the late response agrees with observations in asthmatic subjects (Cockcroft and Murdock, 1987; Durham et al., 1988; Bernstein et al., 1992). Since it has been reported that bronchoconstrictive responses to histamine are partly mediated by cholinergic

activation of the vagus nerve (Holtzman et al., 1980; Hulbert et al., 1985; Santing et al., 1995b), the present results may again be taken as evidence that muscarinic M_2 receptor dysfunction, leading to enhanced acetylcholine release through loss of inhibitory control, contributes to hyperresponsiveness towards histamine after the early but not after the late response. This is supported by the strong inhibition of the histamine hyperresponsiveness after the early reaction by inhalation of ipratropium bromide (Santing et al., 1995b)

In summary, the present study has shown that vagus nerve stimulation-induced bronchoconstriction can be repeatedly induced in conscious, unrestrained guinea pigs in vivo, i.e., without interference of anaesthetics and other drugs, resulting in longer use of the animals as well as in intra-animal comparison of physiological responses. Using this method, it was shown that prejunctional inhibitory muscarinic M_2 autoreceptors are dysfunctional already 6 h after antigen challenge of sensitized guinea pigs, at which moment bronchial responsiveness to histamine is markedly enhanced. This receptor defect is largely restored 24 h after challenge, when histamine bronchial hyperreactivity is clearly diminished. These results indicate that allergen-induced bronchial hyperreactivity to histamine is partly due to muscarinic M_2 receptor dysfunction, resulting in increased acetylcholine release from vagus nerve endings. The application of this model to other pathways of vagally mediated neuronal control of airway diameter, such as excitatory and inhibitory non-adrenergic, non-cholinergic pathways, is currently under investigation.

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