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# Effects of tachykinin $NK_1$ receptor antagonists on vagal hyperreactivity and neuronal $M_2$ muscarinic receptor function in antigen challenged guinea-pigs

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**1** The role of tachykinin $NK_1$ receptors in the recruitment of eosinophils to airway nerves, loss of inhibitory neuronal $M_2$ muscarinic receptor function and the development of vagal hyperreactivity was tested in antigen-challenged guinea-pigs.

**2** In anaesthetized guinea-pigs, the muscarinic agonist, pilocarpine (1–100 $\mu$g kg$^{-1}$, i.v), inhibited vagally induced bronchoconstriction, in control, but not in antigen-challenged guinea-pigs 24 h after antigen challenge. This indicates normal function of neuronal $M_2$ muscarinic receptors in controls and loss of neuronal $M_2$ receptor function in challenged guinea-pigs. Pretreatment of sensitized guinea-pigs with the $NK_1$ receptor antagonists CP99994 (4 mg kg$^{-1}$, i.p.), SR140333 (1 mg kg$^{-1}$, s.c.) or CP96345 (15 mg kg$^{-1}$, i.p.) before antigen challenge, prevented $M_2$ receptor dysfunction.

**3** Neither administration of the $NK_1$ antagonists after antigen challenge, nor pretreatment with an $NK_2$ receptor antagonist, MEN10376 (5 $\mu$mol kg$^{-1}$, i.p.), before antigen challenge, prevented $M_2$ receptor dysfunction.

**4** Electrical stimulation of the vagus nerves caused a frequency-dependent (2–15 Hz, 10 V, 0.2 ms for 5 s) bronchoconstriction that was significantly increased following antigen challenge. Pretreatment with the $NK_1$ receptor antagonists CP99994 or SR140333 before challenge prevented this increase.

**5** Histamine (1–20 nmol kg$^{-1}$, i.v.) caused a dose-dependent bronchoconstriction, which was vagally mediated, and was significantly increased in antigen challenged guinea-pigs compared to controls. Pretreatment of sensitized animals with CP99994 before challenge prevented the increase in histamine-induced reactivity.

**6** Bronchoalveolar lavage and histological studies showed that after antigen challenge significant numbers of eosinophils accumulated in the airways and around airway nerves. This eosinophilia was not altered by pretreatment with the $NK_1$ receptor antagonist CP99994.

**7** These data indicate that pretreatment of antigen-sensitized guinea-pigs with $NK_1$, but not with $NK_2$ receptor antagonists before antigen challenge prevented the development of hyperreactivity by protecting neuronal $M_2$ receptor function. $NK_1$ receptor antagonists do not inhibit eosinophil accumulation around airway nerves.

**Keywords:** Eosinophils; parasympathetic nerves; inflammation

## Introduction

Antigen challenged animals have increased vagally mediated bronchoconstriction (Larsen *et al*., 1994; Santing *et al*., 1995) this is due, in part, to increased release of acetylcholine from the parasympathetic nerves (Larsen *et al*., 1994). Acetylcholine released from these nerves binds to muscarinic $M_3$ receptors on the airway smooth muscle causing contraction and bronchoconstriction (Roffel *et al*., 1990). At the same time, binding of acetylcholine to muscarinic $M_2$ autoreceptors on the parasympathetic nerves inhibits acetylcholine release in controls, but not in antigen-challenged guinea-pigs (Fryer & Maclagan, 1984; Blaber *et al*., 1985; Fryer & Wills-Karp, 1991; Patel *et al*., 1995). This loss of neuronal $M_2$ receptor function may be a mechanism for the hyperreactivity seen after antigen challenge of sensitized animals. A similar loss of $M_2$ receptor function is seen in humans with asthma (Ayala & Ahmed, 1989; Minette *et al*., 1989).

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Both eosinophil major basic protein and eosinophil peroxidase are selective allosteric antagonists at $M_2$ muscarinic receptors (Jacoby *et al*., 1993). *In vivo*, one of these eosinophil proteins may be acting as an endogenous antagonist at neuronal $M_2$ receptors since both heparin and poly-1-glutamate, which neutralize positively charged proteins such as eosinophil major basic protein and eosinophil peroxidase, acutely restore $M_2$ receptor function (Fryer & Jacoby, 1992). Furthermore, pretreatment of guinea-pigs with an antibody against eosinophil major basic protein prevents antigen-induced $M_2$ receptor dysfunction (Evans *et al*., 1997). Eosinophils are closely associated with airway nerves in antigen challenged guinea-pigs, further supporting a role for eosinophil products in the loss of $M_2$ receptor function (Costello *et al*., 1997). Pretreatment with an antibody to either interleukin-5 or to very late activation antigen-4 (VLA-4) has been shown to block selectively the influx of eosinophils into the airways of experimental animals, to prevent the development of hyperreactivity after antigen challenge (Pretolani *et al*., 1994) and to prevent loss of neuronal $M_2$ receptor function (Elbon *et al*., 1995; Fryer *et al*., 1997). Thus eosinophils, and specifically eosinophil major basic protein, appear to be

responsible for the loss of $M_2$ muscarinic receptor function in antigen-challenged guinea-pigs.

In the lungs parasympathetic nerves are closely associated with tachykinin containing nerves as demonstrated by immunohistochemistry, as well as by electrophysiological and pharmacological studies (Lundberg *et al*., 1984; Myers & Undem, 1993). The tachykinins released from these afferent nerves include substance P and neurokinin A, and they exert their effects by acting on neurokinin $NK_{1-3}$ receptors (Regoli *et al*., 1994). Tachykinins acting through $NK_1$ receptors play an important role in mediating inflammatory responses in the airways, for example causing airway mucosal oedema (Bertrand *et al*., 1993), and leukocyte adhesion (Baluk *et al*., 1995). *In vitro*, substance P has a number of effects on eosinophil function including chemoattraction (Numao & Agrawal, 1992; Wiedermann *et al*., 1993), activation (De Simone *et al*., 1987) and degranulation (Kroegel *et al*., 1990; Agarwal *et al*., 1996). Furthermore, 24 h after antigen challenge the numbers of substance P immunoreactive nerves are significantly increased, suggesting that substance P may be important in mediating the inflammatory responses to antigen challenge (Fischer *et al*., 1996). Thus, tachykinin release from sensory nerves may be responsible for the accumulation and activation of eosinophils around efferent cholinergic nerves in the lungs of antigen-challenged guinea-pigs. These experiments were designed to determine whether $NK_1$ receptor antagonists can protect the function of neuronal $M_2$ muscarinic receptors and thus prevent hyperreactivity in antigen-challenged guinea-pigs.

## Methods

Specific pathogen-free guinea-pigs (Dunkin Hartley; 200–250 g) were purchased from Hilltop (Scottdale, PA). Guinea-pigs were shipped in filtered crates and housed in laminar flow hoods in clean rooms. All guinea-pigs were handled in accordance with the standards established by the U.S.A. Animal Welfare Acts set forth in the National Institute of Health guidelines and the Policy and Procedures Manual published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee.

### *Sensitization and challenge*

Guinea-pigs were injected i.p. with 10 mg $kg^{-1}$ ovalbumin on days 1, 3 and 5. Three weeks later the sensitized guinea-pigs were exposed to an aerosol of 5% ovalbumin for 5 min. In some sensitized animals that were not used for histological or bronchoalveolar lavage studies, ovalbumin (250 mg $kg^{-1}$, i.v.) was given to demonstrate that sensitization to ovalbumin had been achieved. In these animals ovalbumin caused a rapid sustained rise in pulmonary inflation pressure to over 600 $mmH_2O$, indicating that the animals had been sensitized to ovalbumin.

### *Tachykinin receptor antagonists*

The $NK_1$ receptor antagonists, CP96345 (15 mg $kg^{-1}$, i.p.), the inactive enantiomer of CP96345, CP96344 (15 mg $kg^{-1}$, i.p.) and CP99994 (4 mg $kg^{-1}$, i.p.), were given 1 h before antigen challenge (Bertrand *et al*., 1993; Baluk *et al*., 1995). The $NK_1$ receptor antagonist SR140333 (1 mg $kg^{-1}$, s.c.) was given 3 h before antigen challenge (Amann *et al*., 1995). The $NK_2$ receptor antagonist MEN10376 (5 $\mu$mol $kg^{-}$, i.p.) was given 1 h before antigen challenge.

### *Measurement of pulmonary inflation pressure*

The experiments were carried out 18–24 h after the exposure of sensitized guinea-pigs to ovalbumin, or for the non-challenged, control group on day 26. The guinea-pigs were anaesthetized with urethane (1.5 mg $kg^{-1}$, i.p.). None of the experiments lasted longer than 3 h, while this dose of urethane produces a deep anaesthesia lasting 8–10 h (Green, 1982). However, because paralyzing agents were used, the depth of anaesthesia was monitored by observing for fluctuations in heart rate and blood pressure.

Once the guinea-pigs were anaesthetized, a carotid artery was cannulated for measurement of blood pressure and heart rate. Cannulae were placed into both jugular veins for the administration of drugs. The animal's body temperature was maintained at 37°C by use of a homeothermic heating blanket (Harvard, Cambridge, MA).

The animals were ventilated with a positive pressure, constant volume animal ventilator (Harvard, Cambridge, MA) and were paralyzed with suxamethonium (infused at 10 $\mu$g $kg^{-1}$ $min^{-1}$). Pulmonary inflation pressure (Ppi) was measured with a pressure transducer (Spetromed DTX, Oxnard, C.A.). All signals were displayed on a Grass polygraph (Quincy, MA).

A positive pressure of 100–150 $mmH_2O$ was needed for adequate ventilation of the animals. Bronchoconstriction was measured as the increase in Ppi over the basal inflation pressure produced by the ventilator (Dixon & Brody, 1903; Blaber *et al*., 1985). The sensitivity of the method was increased by taking the output Ppi signal from the driver to the input of the preamplifier of a second channel on the polygraph. Thus Ppi was recorded on one channel and increases in Ppi were recorded on a separate channel at a greater amplification. By use of this method increases in pressure as small as 2–3 $mmH_2O$ could be recorded accurately.

The sympathetic nerves in the neck lie close to the vagus nerves and since these nerves functionally inhibit cholinergic nerve transmission, guanethidine (10 mg $kg^{-1}$, i.v.) was given to deplete noradrenaline (Blaber *et al*., 1985), before all experiments. Guanethidine temporarily reduced the magnitude of vagally-induced bronchoconstriction and bradycardia. Thirty minutes after guanethidine administration when the heart rate and blood pressure had returned to baseline the experiments were performed.

### In vivo *measurement of $M_2$ muscarinic receptor function*

Both vagi were cut and the distal portions placed on shielded electrodes immersed in a pool of liquid paraffin. Both vagus nerves were simultaneously stimulated at 1 min intervals (2 Hz, 0.2 ms, 10–50 V, 44 pulses per train). Two Hz was chosen since the effects of muscarinic agonists on the $M_2$ receptors are more readily apparent at low frequencies of stimulation (Fryer & Maclagan, 1984). The degree of vagally induced bronchoconstriction in the absence of pilocarpine was matched between groups by varying the voltages between 10–50 V. Thirty minutes after guanethidine, when the responses to stimulation of the vagus nerves were back to pre-guanethidine values and were reproducible, the effect of pilocarpine (1–100 $\mu$g $kg^{-1}$, i.v.) on vagally-induced bronchoconstriction was determined. At the end of each experiment vagally-induced bronchoconstriction and bradycardia were abolished by atropine (1 mg $kg^{-1}$, i.v.), indicating that both of these responses were mediated via muscarinic receptors.

The effect of pilocarpine on the response to vagal stimulation was calculated as the ratio of the change in Ppi in response to vagal stimulation with no drug compared to that occurring after each dose of pilocarpine.

### *Vagal nerve hyperreactivity*

The distal ends of the vagus nerves were attached to shielded electrodes and simultaneously stimulated at 90 s intervals. The voltage (10 V), the stimulus train (5 s) and pulse duration (0.2 ms) were kept constant while the frequency was increased from 2–15 Hz. The peak rise in pulmonary inflation pressure above baseline in response to increasing frequency of vagal nerve stimulation was recorded and compared between groups of animals.

### *Histamine hyperreactivity*

Thirty minutes after the administration of guanethidine when the heart rate and blood pressure had returned to baseline, increasing doses of histamine sulphate (1–20 nmol kg$^{-1}$, i.v.) were given at five minute intervals. The rise in pulmonary inflation pressure above baseline was recorded and compared between groups. The vagus nerves were then cut and the dose-response curves to histamine (1–20 nmol kg$^{-1}$, i.v.) were repeated.

### *Bronchoalveolar lavage*

In some animals, after the *in vivo* studies had been completed bronchoalveolar lavage was performed *in situ* via the tracheal cannula. The lungs were lavaged with 5 aliquots of 10.0 ml phosphate buffered saline (PBS). The recovered lavage fluid was centrifuged, the supernatant poured off and the cells resuspended in 10 ml of PBS. The total cell number was counted under a Neubauer haemocytometer (Hausser Scientific, Hoarsham, PA). Aliquots of the cell suspension were cytospun onto glass slides, stained with Diff-Quick (Baxter Healthcare Corp., McGaw Park IL) and cells were counted.

### *Histological preparations*

In other animals, at the end of the *in vivo* studies while the animals were under deep anaesthesia the thorax of the guinea-pig was opened, a cannula inserted into the portal vein and the animal exsanguinated via the abdominal aorta by perfusing the portal vein with PBS, 100 ml at 60 mmHg for one minute. After exsanguination, the lungs were fixed by infusing 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer at 60 mmHg over a 5 min period. The trachea and lungs were removed, the lungs inflated gently to approximately functional residual capacity (10–15 mg kg$^{-1}$) with 4% paraformaldehyde and fixed in this manner at 4°C for 48 h. Sections from each lobe were cut, embedded in paraffin and 6 $\mu$m thick sections were cut and placed on positively charged slides.

### *Identification of airway nerves by use of PGP 9.5*

The polyclonal rabbit antibody to protein gene product 9.5 (PGP 9.5) (Wilson *et al*., 1988) was used to identify airway nerves. Sections of paraffin embedded tissue were dewaxed by immersion in xylene, rehydrated through a graded alcohol series and washed three times in Tris HCl (pH = 7.8). Sections were then placed in a solution of Tissue Unmasking Fluid at 90°C for 10 min and then brought to room temperature over 10 min and washed in Tris HCl. Non-specific peroxidase activity was inhibited by applying 3% hydrogen peroxide in methanol for 10 min. The tissue was then overlaid with 10% normal goat serum for 30 min and rabbit anti-PGP 9.5 (1 : 4,000) was applied to the tissue for 36 h at 4°C. The tissue was washed three times in Tris HCl over 15 min and a biotinylated goat anti-rabbit antibody was added for 30 min. The tissue was rewashed and an avidin horseradish peroxide complex was added for 30 min. The antibody was detected by the addition of the chromagen SG in the presence of hydrogen peroxide. Rabbit serum absorbed over PGP 9.5 was used as a control antibody. To identify eosinophils the sections were stained in a solution of 1% Chromotrope 2R for 30 min. The sections were then dehydrated, washed in toulene and mounted under a coverslip.

### *Histological analysis*

Four cartilaginous bronchi from each of 5 animals representing each experimental group were examined (20 airways per group). Airways were selected by starting at the top left corner of the slide and moving in a counter clockwise manner. An obvious landmark within an airway was chosen under low power as the starting point for analysis. Beginning at this point, the sections were viewed under an oil immersion lens, this image was captured to a video camera attached to the microscope and relayed to a viewing screen. The imaged was oriented so that the basement membrane was uppermost, thus, a section of airway containing mucosa and airway smooth muscle was examined. Ten consecutive sections were examined. The number of eosinophils within 8 $\mu$m of a PGP 9.5 immunoreactive nerve and the total number of eosinophils were counted in each section. The area examined was calculated by use of an image analysis programme (Image Pro Plus). In order to ensure that the airways were of the same size between groups the airway wall area was also measured.

### *Drugs*

Atropine, chromotrope 2R, guanethidine, histamine, ovalbumin, pilocarpine, suxamethonium, and urethane were purchased from Sigma (St. Louis, MO, U.S.A.). MEN10376 H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH2 was purchased from RBI (Natick, MA, U.S.A.). CP96345 ((2**S**,3**S**-[*cis*-2-diphenylmethyl)-N-[2-methoxyphenyl)-methyl]-1-azabicyclo [2.2.2]-octan-3-amine]), CP96344 ((2**R**,3**R**-[*cis*-2-(diphenylmethyl)-N-[2-methoxyphenyl)-methyl)-1-azabicyclo [2,2,2]octan-3-amine) and CP99994 ((+),-(2**S**,3**S**)-3-(2-methoxybenzylamino)-2-phenylipiperidine) were a generous gift from Dr Saul Kadrin, Pfizer Inc (Groton Ct, U.S.A.). SR14033 ((**S**)1-{2-[3-(3,4-dichlorophenyl)-193-isopropoxyphenylacetyl(piperidin-n-3-y]ethyl}-4-phenyl-1azoniabi-cyclo[2,2,2]octane chloride), was a generous gift from Dr X. Emonds-Alt, Sanofi Recherche (Montpellier, France). PGP 9.5 and immune absorbed PGP 9.5 serum was purchased from Biogenesis (Sandown, NH). Goat serum, biotinylated anti-rabbit antibody, ABC 'elite' and the chromagen SG were all purchased from Vector (Burlingame, CA). All drugs were dissolved and diluted in 0.9% NaCl, except SR140333 which was dissolved in dimethylsulphoxide (DMSO, 10 mg ml$^{-1}$, stock).

### *Statistics*

All data are expressed as a mean ± s.e.mean. Baseline pulmonary inflation pressure, heart rate, blood pressure, the rise in pulmonary inflation pressure in response to vagal stimulation, the numbers of inflammatory cells in the

bronchoalveolar lavage fluid, the number of eosinophils per $mm^2$ within the airway wall and around airway nerves were compared between groups of animals by an analysis of variance (ANOVA). The rise in pulmonary inflation pressure in response to histamine, the frequency-dependent changes in pulmonary inflation pressure in response to vagal nerve stimulation and the effect of pilocarpine on vagally induced bronchoconstriction, pulmonary inflation pressure and heart rate were compared between groups by means of a two way analysis of variance for repeated measures with a *post hoc* correction for repeated measures with the Bonferroni-Dunn method (Statview 4.5, Abacus Concepts, Inc., Berkeley, CA). A $P$ value less than 0.05 was considered significant.

## Results

Baseline Ppi, blood pressure and heart rate were the same in control, antigen challenged and antigen challenged guinea-pigs treated with CP96346, CP99994, MEN10376 and SR140333; the values for each group are shown in Table 1.

### *Effects of $NK_1$ receptor antagonists on the ability of pilocarpine to inhibit vagally-induced bronchoconstriction*

Simultaneous electrical stimulation of both vagus nerves (2 Hz, 0.2 ms, 10–50 V, 45 pulses/train) caused bradycardia and bronchoconstriction. The voltages used to stimulate the vagus nerves electrically were varied (10–50 V) so that the degree of vagally-induced bronchoconstriction, measured as a rise in Ppi was kept as close as possible to 20 $mmH_2O$ in each animal. The degree of vagally-induced bronchoconstriction was not significantly different between any of the groups (Table 1). Both bronchoconstriction and bradycardia were transient and readily reversed after electrical stimulation was stopped. Both were also completely blocked by atropine (1 mg $kg^{-1}$, i.v.), indicating that they were mediated by the release of acetylcholine onto muscarinic receptors.

In control guinea-pigs the muscarinic agonist pilocarpine (1–100 $\mu$g $kg^{-1}$, i.v.) inhibited vagally-induced bronchoconstriction in a dose-dependent manner (Figure 1; $n=7$). In contrast, pilocarpine had no effect on vagally-induced bronchoconstriction in antigen-challenged guinea-pigs (Figure 1; $n=7$). The difference in response to pilocarpine between challenged and control animals was significant, $P=0.0001$. In antigen-challenged guinea-pigs that had been pretreated with the $NK_1$ receptor antagonists CP99994 (Figure 1; $n=5$), or SR140333 (Figure 1; $n=5$) or CP96345 (Figure 1; $n=6$) but not with the inactive enantiomer of CP96345, CP96344 (data not shown, $n=3$), before antigen challenge, pilocarpine caused a dose-dependent fall in vagally-induced bronchoconstriction. The effect of pilocarpine in $NK_1$ receptor antagonist-pretreated guinea-pigs was not significantly different from the effect of pilocarpine in control guinea-pigs but was significantly different from the effect of pilocarpine in antigen-challenged guinea-pigs (compared to antigen challenged guinea-pigs, all $NK_1$ receptor antagonists, $P<0.001$).

### *Effects of a $NK_2$ receptor antagonist on the ability of pilocarpine to inhibit vagally-induced bronchoconstriction*

In antigen challenged guinea-pigs pretreated with the $NK_2$ receptor antagonist, MEN10376 pilocarpine did not inhibit vagally-induced bronchoconstriction (Figure 2; $n=4$). The responses to pilocarpine in these animals was not different

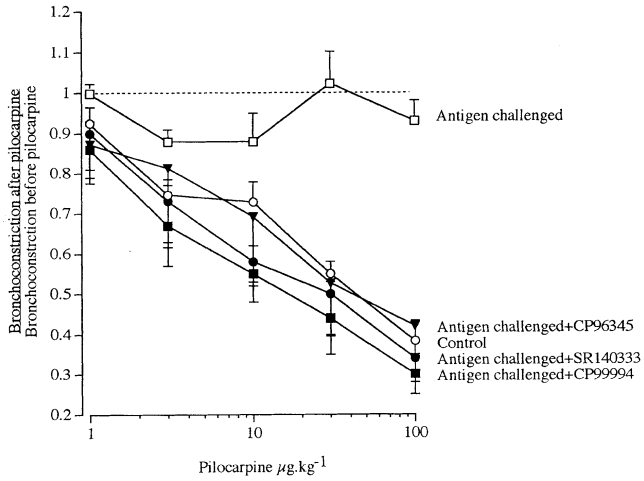


**Figure 1** Pilocarpine (1–100 $\mu$g $kg^{-1}$, i.v.) inhibited vagally-induced bronchoconstriction in control guinea-pigs ($n=7$) but not in antigen-challenged guinea-pigs ($n=7$). Pretreatment of sensitized guinea-pigs with the neurokinin $NK_1$ receptor antagonists CP96345 (15 mg $kg^{-1}$, i.p.; $n=6$), CP99994 (4 mg $kg^{-1}$, i.p.; $n=5$) or SR140333 (1 mg $kg^{-1}$, s.c.; $n=5$) before antigen challenge protected the ability of pilocarpine to inhibit vagally-induced bronchoconstriction. There was a significant difference between antigen-challenged animals and control guinea-pigs ($P=0.001$) and also between challenged animals and guinea-pigs pretreated with the $NK_1$ receptor antagonists (compared to antigen challenged animals all $P<0.001$). Values are mean and vertical lines show s.e.mean.

**Table 1** Baseline pulmonary inflation pressure, blood pressure, heart rate, rise in pulmonary inflation pressure in response to electrical stimulation of the vagus nerves (B1) and the mean voltage used to induce B1

| | *Baseline Ppi* ($cmH_20$) | *Baseline heart rate* (beats $min^{-1}$) | *Systolic pressure* (mmHg) | *Diastolic pressure* (mmHg) | *B1* ($cmH_20$) | *Voltage* (V) |
|---|---|---|---|---|---|---|
| Control | 102±10 | 287±4 | 49±5 | 30±3 | 20.3±2 | 24±3 |
| Antigen challenged | 116±4 | 286±23 | 45±3 | 23±5 | 21.2±3.4 | 30±2 |
| Pretreatment before antigen challenge | | | | | | |
| CP99994 | 88±10 | 290±21 | 51±1 | 28.5±6 | 21.9±2.4 | 19±2 |
| CP96345 | 120±6 | 260±12 | 53±4 | 24±2 | 24.3±0.4 | 25±6 |
| SR140333 | 84±6 | 260±12 | 54±11 | 30±5 | 24.3±0.4 | 19±6 |
| MEN10376 | 105±5 | 300±22 | 54±4 | 23±1 | 28.3±0.4 | 34±13 |
| Treatment after antigen challenge | | | | | | |
| CP96345 | 116±4 | 294±13 | 50±3 | 22±2 | 21.9±1.4 | 32±13 |

Data shown are means±s.e.mean.

from those of the untreated antigen-challenged guinea-pigs, but was significantly different from the controls (Figure 2, $P=0.01$).

### *Effects of administration of a NK$_1$ receptor antagonist after antigen challenge on the ability of pilocarpine to inhibit vagally-induced bronchoconstriction*

Administration of CP96345 (1 mg kg$^{-1}$, i.v.) to guinea-pigs 24 h post antigen challenge reduced vagally-induced bronchoconstriction by $52\pm4.0\%$. However, in contrast to the effects of CP96345 when given before antigen challenge, CP96345 administered 24 h after antigen challenge did not restore the ability of pilocarpine to inhibit vagally-induced bronchoconstriction (Figure 3) compared to untreated controls ($P=0.03$).

In unchallenged, control animals, administration of CP96345 (1 mg kg$^{-1}$, i.v.), 30 min before $M_2$ muscarinic receptor function was tested, also reduced vagally-induced bronchoconstriction by $43\pm3.3\%$. Pilocarpine (1–100 $\mu$g kg$^{-1}$, i.v.) subsequently induced vagally-induced bronchoconstriction in a dose-dependent manner in these animals (data not shown, $n=2$).

### *Effects of pilocarpine on postjunctional M$_2$ and M$_3$ receptors*

In addition to its effects on vagally-induced bronchoconstriction, pilocarpine caused transient bradycardia and bronchoconstriction (Figure 4a and b) via stimulation of $M_2$ receptors in the heart and $M_3$ receptors on the airway smooth muscle. Pilocarpine-induced bronchoconstriction was not different between any of the groups of animals tested. Pilocarpine induced bradycardia was significantly greater in CP99994 pretreated animals than in either SR140333-pretreated animals or control animals ($P=0.02$).

### *Effects of NK$_1$ receptor antagonists on vagal nerve hyperreactivity*

Electrical stimulation of the vagus nerves (10 V, 0.2 ms, 5 s duration, 2–15 Hz) caused a frequency-dependent rise in vagally-induced bronchoconstriction in control animals (Figure 5; $n=5$). In antigen challenged animals (Figure 5; $n=4$) the frequency-dependent rise in vagally-induced bronchoconstriction was significantly increased compared to controls

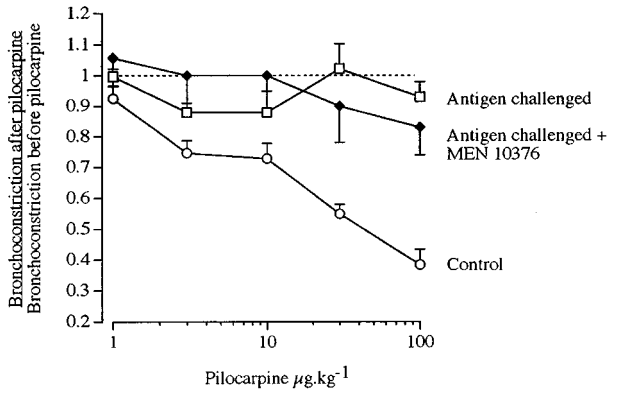


**Figure 2** Pilocarpine (1–100 $\mu$g kg$^{-1}$, i.v.) inhibited vagally-induced bronchoconstriction in control guinea-pigs ($n=7$) but not in antigen-challenged guinea-pigs ($n=7$). Pilocarpine did not inhibit vagally-induced bronchoconstriction in antigen-sensitized guinea-pigs pretreated with the neurokinin NK$_2$ receptor antagonist, MEN10376 (5 $\mu$mol kg$^{-1}$, i.p.), 1 h before antigen challenge ($n=4$). Values are mean and vertical lines show s.e.mean.

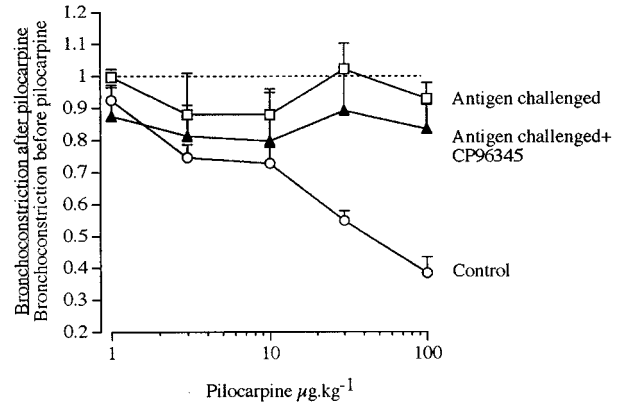


**Figure 3** Pilocarpine (1–100 $\mu$g kg$^{-1}$, i.v.) inhibited vagally-induced bronchoconstriction in control guinea-pigs ($n=7$) but not in antigen-challenged guinea-pigs ($n=7$). Administration of the NK$_1$ receptor antagonist CP96345 (1 mg kg$^{-1}$, i.v.) to antigen-challenged guinea-pigs 24 h after challenge and 30 min before testing $M_2$ receptor function did not restore the ability of pilocarpine to inhibit vagally-induced bronchoconstriction ($n=4$). Values are mean and vertical lines show s.e.mean.

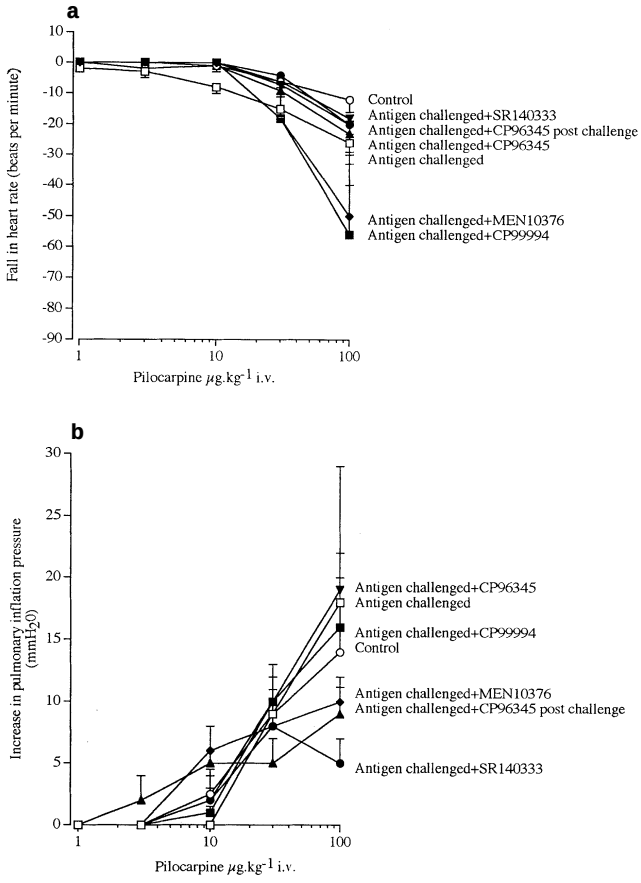


**Figure 4** Pilocarpine caused a dose-dependent fall in heart rate (a) and rise in Ppi (b), in control guinea-pigs ($n=5$), antigen-challenged guinea-pigs ($n=5$), CP96345-pretreated guinea-pigs ($n=5$), MEN10376-pretreated guinea-pigs ($n=4$), in guinea-pigs acutely treated with CP96345 ($n=4$), sensitized guinea-pigs pretreated with CP99994 before challenge ($n=4$) and sensitized guinea-pigs pretreated with SR140333 ($n=5$) before challenge. The fall in heart rate was significantly greater in the CP99994 group than in the other groups of guinea-pigs. There was no difference in the rise in Ppi between any of the groups of animals. Values are mean and vertical lines show s.e.mean.

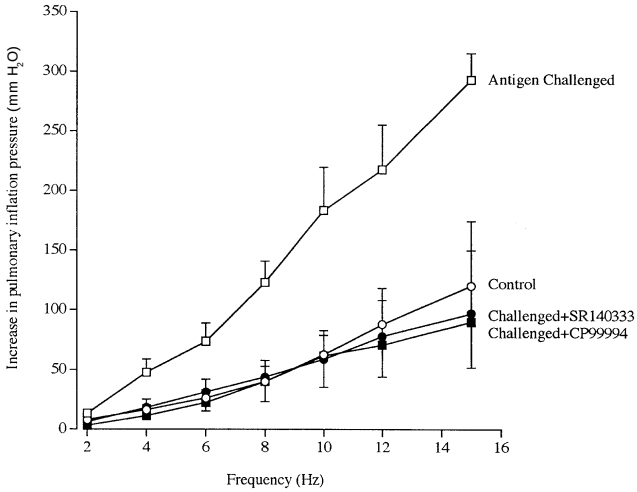


**Figure 5** Stimulation of the vagus nerves caused a frequency-dependent increase in pulmonary inflation pressure that was significantly increased in antigen-challenged guinea-pigs ($n=4$) compared to control animals ($n=5$; $P=0.001$). Pretreatment of antigen-sensitized guinea-pigs with the neurokinin $NK_1$ receptor antagonist CP99994 (4 mg $kg^{-1}$, i.p.; $n=4$) or SR140333 (1 mg $kg^{-1}$, s.c.; $n=4$), before antigen challenge prevented vagally-induced hyperreactivity. Values are mean and vertical lines show s.e.mean.

($P=0.001$). Pretreatment of antigen-sensitized animals with the $NK_1$ receptor antagonists SR140333 ($n=4$) or CP99994 ($n=4$) before challenge prevented the increase in response to electrical stimulation of the vagus nerves (Figure 5; compared to challenged only animals, both $P<0.001$).

### *Effects of $NK_1$ receptor antagonist on hyperreactivity to histamine*

Administration of histamine (1–20 nmol $kg^{-1}$, i.v.) caused a dose-dependent increase in pulmonary inflation pressure in control animals (Figure 6a; $n=6$). The response to histamine was significantly increased in antigen-challenged animals (Figure 6a; $n=5$), compared to controls ($P=0.001$). Pretreatment of antigen-sensitized animals with the $NK_1$ receptor antagonist CP99994 ($n=4$) before challenge prevented the increased response to histamine (Figure 6a; compared to antigen challenged only animals, $P<0.01$). When the vagus nerves were cut and histamine (1–20 nmol $kg^{-1}$, i.v.) administered, the rise in pulmonary inflation pressure was the same in all groups of animals (Figure 6b).

### *Effects of a $NK_1$ receptor antagonist on eosinophil influx following antigen challenge*

Compared to control animals, there was a significant increase in the number of eosinophils in the airway wall of antigen-challenged guinea-pigs (Figure 7; $P=0.0001$). The number of eosinophils associated with the airway nerves of antigen challenged guinea-pigs was also significantly greater than in control animals (Figure 7; $P=0.0001$). Pretreatment with the $NK_1$ receptor antagonist CP99994 before antigen-challenge did not inhibit the number of eosinophils in the airways. They were further increased so that the number of eosinophils in the whole airway wall and in association with the nerves of challenged guinea-pigs treated with CP99994 was higher even than antigen-challenged only animals (Figure 7; $P<0.01$).

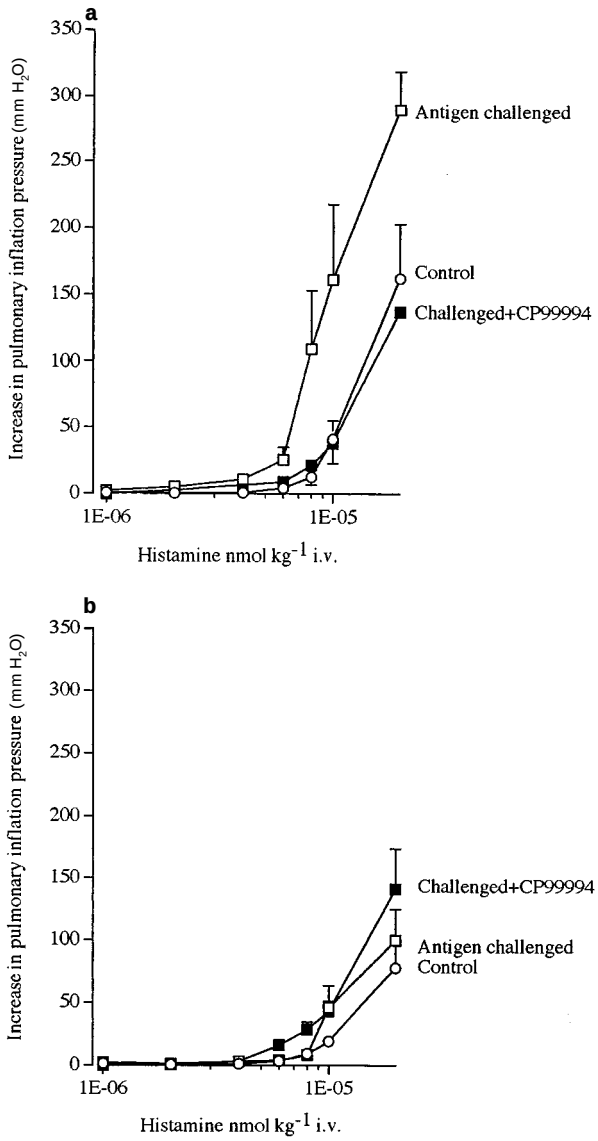


**Figure 6** Histamine (1–20 nmol $kg^{-1}$, i.v.) caused a dose-dependent increase in pulmonary inflation pressure in all animals. The response to histamine was significantly increased in antigen-challenged guinea-pigs ($n=5$) compared to control animals ($n=6$) when the vagus nerves were intact (a), but not when the nerves were cut (b). Pretreatment with the $NK_1$ receptor antagonist CP99994 (4 mg $kg^{-1}$, i.p.) before antigen challenge prevented the increased reactivity to histamine when the vagus nerves were intact and did not affect histamine-induced bronchoconstriction when the vagus nerves were cut ($n=4$). Values are mean and vertical lines show s.e.mean.

### *Effects of pretreatment with a $NK_1$ receptor antagonist on inflammatory cell influx into the bronchoalveolar fluid following antigen challenge*

Compared to control animals there was a significant increase in the number of macrophages, lymphocytes and eosinophils recovered by bronchoalveolar lavage in antigen challenged animals (Figure 8). Pretreatment of antigen sensitized animals with the $NK_1$ receptor antagonist SR140333 significantly reduced the number of total cells, macrophages and lymphocytes recovered by bronchoalveolar lavage compared to antigen challenged only animals (Figure 8). Pretreatment of antigen sensitized animals with the $NK_1$ receptor antagonist SR140333 did not significantly inhibit the number of eosinophils recovered in the bronchoalveolar lavage.

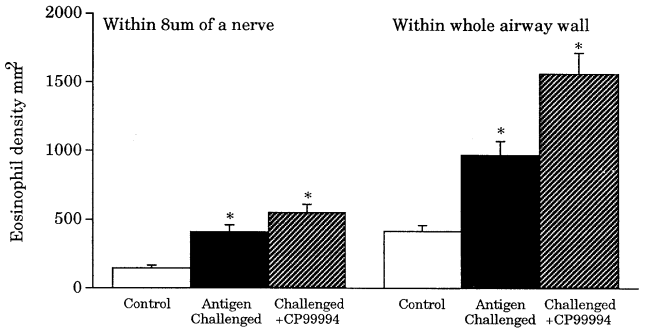


**Figure 7** The $NK_1$ receptor antagonist CP99994 (4 mg $kg^{-1}$, i.p.) did not prevent eosinophil accumulation following antigen challenge. The eosinophil density (number of eosinophils/$mm^2$) in a section of airway wall containing mucosa and smooth muscle is shown on the right. The density of eosinophils with 8 $\mu$m of a nerve in this section of airway wall is shown on the left. Compared to controls, there was a significant increase in eosinophil density following antigen challenge in the whole airway wall ($*P<0.01$) and around the airway nerves ($*P<0.01$). The accumulation of eosinophils in the antigen-challenged guinea-pigs was not inhibited by the $NK_1$ receptor antagonist CP99994 and was significantly greater than untreated antigen-challenged animals. Values are mean$\pm$s.e.mean; each group represents the data from 4 airways from each of 5 animals.

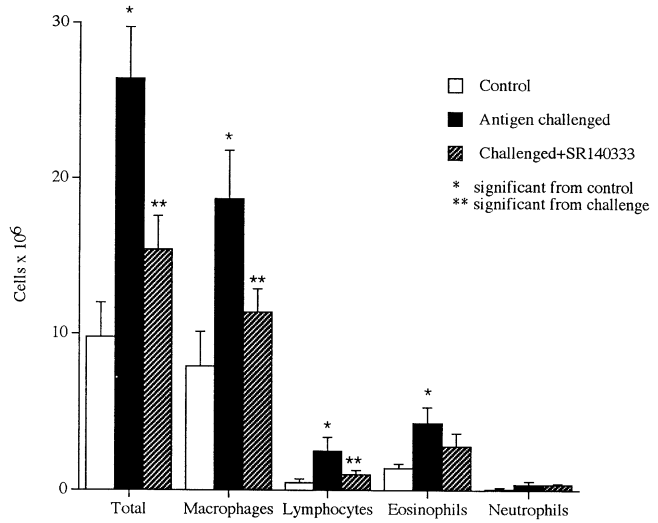


**Figure 8** There was a significant increase in all inflammatory cells except neutrophils recovered by bronchoalveolar lavage of antigen-challenged guinea-pigs ($n=6$) compared to controls ($n=6$). Although pretreatment of guinea-pigs with the neurokinin $NK_1$ receptor antagonist SR140333 (1 mg $kg^{-1}$, s.c.; $n=6$) before antigen challenge inhibited other cell types in the lavage, there was no inhibition of eosinophils recovered by bronchoalveolar lavage compared to antigen-challenged only animals. Values are mean$\pm$s.e.mean. $*P<0.001$, significantly different from control; $**P=0.001$, significantly different from challenged.

## Discussion

In control guinea-pigs, the muscarinic agonist pilocarpine inhibited vagally-induced bronchoconstriction indicating the presence of functional neuronal $M_2$ receptors (Fryer & Maclagan, 1984). Pilocarpine did not inhibit vagally-induced bronchoconstriction in sensitized and challenged guinea-pigs, indicating decreased $M_2$ muscarinic receptor function in these animals. These results confirm previous findings in control and antigen challenged guinea-pigs (Fryer & Jacoby, 1992; Elbon *et al.*, 1995).

Pretreatment with the $NK_1$ receptor antagonists CP99994, SR140333 or CP96345 prevented loss of function of neuronal $M_2$ muscarinic receptors in antigen-sensitized and challenged guinea-pigs (Figure 1). In contrast, pretreatment of sensitized guinea-pigs with the inactive enantiomer CP96344 or the $NK_2$ receptor antagonist MEN10376 did not preserve the function of $M_2$ receptors following antigen challenge, indicating a specific $NK_1$ receptor effect (Figure 2). This protection of neuronal $M_2$ receptor function was dependent on treatment before antigen challenge, since administration of CP96345 24 h after antigen challenge (30 min before testing receptor function) gave no protection (Figure 3). Thus, only pretreatment with the $NK_1$ receptor antagonists could protect the neuronal $M_2$ muscarinic receptors from antigen challenge.

The fall in heart rate with pilocarpine, which is mediated via $M_2$ muscarinic receptors on the heart, was the same in all groups of animals except those treated with CP99994. Since the other $NK_1$ receptor antagonists (CP96345 and SR140333) did not likewise increase the fall in heart rate this effect appears to be isolated to this one compound and is difficult to interpret. However, since the heart rate did fall, the cardiac receptors were functional in antigen challenged guinea-pigs. Thus loss of $M_2$ receptor function after antigen challenge was specific for the airways (Figure 4a). Pilocarpine (10–100 $\mu$g $kg^{-1}$, i.v.) caused a transient bronchoconstriction that was the same in the control, antigen challenged animals as well as antigen challenged guinea-pigs pretreated with the $NK_1$ and $NK_2$ receptor antagonists, indicating normal function of the postjunctional $M_3$ muscarinic receptors (Figure 4b). These results confirm previous findings in control and antigen-challenged guinea-pigs (Fryer & Jacoby, 1992; Elbon *et al.*, 1995; Fryer *et al.*, 1997). Thus, the postjunctional $M_3$ receptors in the lungs were not altered by antigen challenge nor by the tachykinin receptor antagonists.

Since loss of functional neuronal $M_2$ muscarinic receptors leads to increased release of acetylcholine from postganglionic nerves it should be expected that stimulation of the vagus nerves in these animals would lead to heightened contractile responses to vagal nerve stimulation. This was demonstrated in antigen-challenged guinea-pigs by showing that the frequency-dependent changes in vagally-induced bronchoconstriction were increased compared to control animals. Increased vagal nerve function in antigen-challenged animals compared to control animals was also demonstrated by the increased contractile response to histamine when the vagus nerves were intact. Cutting the vagus nerves resulted in the loss of significant differences in the response to histamine in antigen-challenged animals, indicating that histamine-induced hyperreactivity is vagally mediated. These results confirm previous findings that antigen-induced hyperreactivity is vagally mediated (Santing *et al.*, 1995; Fryer *et al.*, 1997; Evans *et al.*, 1997). Pretreatment with the $NK_1$ receptor antagonists CP99994 or SR140333 prevented the development of hyperreactivity in antigen-challenged animals. Thus, by preventing the loss of neuronal $M_2$ muscarinic receptor function in antigen-challenged guinea-pigs the $NK_1$ receptor antagonists prevented the development of vagal nerve hyperreactivity.

The mechanism of protection of neuronal $M_2$ muscarinic receptor function was then investigated by assessing if there was a direct interaction between the neuronal $M_2$ muscarinic receptors and $NK_1$ receptor antagonists. The possibility of a direct interaction between the $NK_1$ receptor antagonist and $M_2$ receptors was tested by giving the $NK_1$ receptor antagonist CP96345 24 h after antigen challenge, 30 min before testing the function of the $M_2$ receptors. In these experiments there

was no protection of $M_2$ receptor function by the $NK_1$ receptor antagonist (Figure 3). Thus, it is unlikely that pretreatment with $NK_1$ receptor antagonists protects $M_2$ receptor function by a direct interaction with the neuronal $M_2$ muscarinic receptors.

Since the $NK_1$ receptor antagonists protect $M_2$ receptor function only when given before antigen challenge, this suggested that the $NK_1$ receptor antagonists were acting indirectly by inhibiting the influx or activation of inflammatory cells. Eosinophil accumulation around airway nerves and the release of eosinophil major basic protein mediates the loss of function of neuronal $M_2$ muscarinic receptors. Therefore, it is possible that the $NK_1$ receptor antagonists are protecting the function of neuronal $M_2$ muscarinic receptors, either by inhibiting antigen-induced eosinophil influx or by inhibiting eosinophil degranulation. There is considerable evidence to suggest that tachykinins acting through $NK_1$ receptors could play an important role in promoting the accumulation of eosinophils into the airways after antigen challenge. Substance P is an eosinophil chemoattractant *in vitro* (Numao & Agrawal, 1992; Weidermann *et al*., 1993) and the $NK_1$ receptor antagonist CP96345 can inhibit neurogenic eosinophil inflammation, *in vivo* (Bertrand *et al*., 1993).

Eosinophil density around the airway nerves was increased in antigen-challenged guinea-pigs compared to controls, confirming the results of previous studies (Costello *et al*., 1997; Evans *et al*., 1997). Pretreatment with the $NK_1$ receptor antagonists did not inhibit antigen-induced eosinophil accumulation around the airway nerves. This suggests that the accumulation of eosinophils around these structures is not mediated by tachykinins. This finding is in agreement with another study that showed that capsaicin depletion before antigen challenge did not prevent the influx of eosinophils into the airways, but did prevent hyperreactivity to acetylcholine (Matsuse *et al*., 1991).

Eosinophil adherence to post capillary venules and the subsequent migration into the lungs following neurogenic stimulation is mediated via $NK_1$ receptors (Baluk *et al*., 1995). $NK_1$ receptors increase intracellular calcium in endothelial cells and promote the expression of the adhesion molecule P-selectin (McEver *et al*., 1989). In contrast to this response to a neurogenic stimulus we and others have shown that antigen-induced influx of eosinophils depends on binding of eosinophil VLA-4 to the endothelial cell adhesion molecule vascular adhesion molecule-1 (VCAM-1) (Pretolani *et al*., 1994; Fryer *et al*., 1997). While tachykinins increase P-selectin expression, substance P does not alter the expression of VCAM-1 (Smith *et al*., 1993), which may explain why pretreatment with the $NK_1$ receptor antagonists did not inhibit eosinophil influx in the airways of antigen-challenged guinea-pigs as they did following neurogenic stimulation (Bertrand *et al*., 1993).

The $NK_1$ receptor antagonists may have prevented antigen-induced loss of neuronal $M_2$ muscarinic receptor function by inhibiting eosinophil activation or degranulation. Tachykinins acting in part through $NK_1$ receptors have been shown to active both IgG and IgE surface receptor expression on eosinophils and also to promote eosinophil degranulation (De Simone *et al*., 1987; Kroegel *et al*., 1990; Iwamoto *et al*., 1993a; Elshazly *et al*., 1996; Agarawal *et al*., 1996). Tachykinins may also alter eosinophil function indirectly by producing agents that act directly on eosinophils. Tachykinins acting through $NK_1$ receptors stimulate the production of leukotrienes, as well as proinflammatory cytokines such as interleukin 2, interleukin 3, granulocyte-macrophage colony stimulating factor and tumour necrosis factor $\alpha$ (Ansel *et al*., 1993; Iwamoto *et al*., 1993b; Rameshwar *et al*., 1993; Walsh *et al*., 1995). All of these cytokines have been shown to promote eosinophil activation or degranulation. Thus, it is possible that the $NK_1$ receptor antagonists protected $M_2$ muscarinic receptors after antigen challenge either by acting directly or indirectly on eosinophils to inhibit the release of eosinophil major basic protein. Major basic protein is a known $M_2$ muscarinic receptor antagonist (Jacoby *et al*., 1993), and we have shown that pretreating guinea-pigs with an antibody to this protein prevents antigen-induced $M_2$ receptor dysfunction (Evans *et al*., 1997).

Although it is clear that the eosinophil is responsible for loss of $M_2$ receptor function after antigen challenge, it is also possible that the effects of the $NK_1$ antagonists involve other inflammatory cells. Both macrophages and lymphocytes are responsive to tachykinins (Brunelleschi *et al*., 1990; Rameshwar *et al*., 1993), and our data show decreases in the recruitment of both cell types in $NK_1$ antagonist-treated animals. Defining the role of these cells in $M_2$ receptor dysfunction, and in eosinophil recruitment and activation, will require further studies.

The finding that only pretreatment with the $NK_1$ receptor antagonists could prevent hyperreactivity are in contrast to those obtained by Boichot *et al.* (1995). In their study the authors found that the $NK_2$ receptor antagonist SR48968 but not the $NK_1$ receptor antagonist SR140333 prevented hyperreactivity to acetylcholine. We did not observe hyperreactivity to acetylcholine in antigen-challenged, vagotomized guinea-pigs (unpublished data). However it appears that the vagi were intact in the study by Boichot, so it is difficult to compare these results. In addition, the animals in the study by Boichot were paralyzed with pancuronium, which is an antagonist for $M_2$ muscarinic receptors (Fryer & Maclagan, 1987), thus $M_2$ receptor function would be lost even in their control animals, again making it difficult to compare these two studies.

In conclusion, pretreatment with an $NK_1$ but not with an $NK_2$ receptor antagonist protected the function of the $M_2$ muscarinic receptors and prevented vagally-mediated hyperreactivity in antigen-challenged guinea-pigs. Although the mechanism of protection is unclear, the $NK_1$ receptor antagonists did not inhibit antigen-induced eosinophil accumulation around airway nerves and did not directly influence the function of the neuronal $M_2$ muscarinic receptors. The $NK_1$ receptor antagonists may have protected neuronal $M_2$ muscarinic receptors by inhibiting tachykinin-induced eosinophil activation or degranulation.

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