

# Parainfluenza virus infection damages inhibitory M<sub>2</sub> muscarinic receptors on pulmonary parasympathetic nerves in the guinea-pig

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**1** The effect of viral infection on the function of neuronal M<sub>2</sub> muscarinic autoreceptors in the lungs was studied in anaesthetized guinea-pigs.

**2** Guinea-pigs were inoculated intranasally with either parainfluenza type 3 or with a vehicle control. Four days later the animals were anaesthetized, paralysed and artificially ventilated. Pulmonary inflation pressure, tidal volume, blood pressure, and heart rate were recorded. Both vagus nerves were cut and electrical stimulation of the distal portions caused bronchoconstriction (measured as an increase in pulmonary inflation pressure) and bradycardia.

**3** In control animals, pilocarpine (1–100 µg kg<sup>-1</sup>, i.v.) attenuated vagally-induced bronchoconstriction by stimulating inhibitory M<sub>2</sub> muscarinic receptors on parasympathetic nerves in the lungs. Conversely, blockade of these receptors with the antagonist gallamine (0.1–10 mg kg<sup>-1</sup>, i.v.) produced a marked potentiation of vagally-induced bronchoconstriction. These results confirm previous findings.

**4** In guinea-pigs infected with parainfluenza virus, pilocarpine did not inhibit vagally-induced bronchoconstriction. Furthermore, gallamine did not potentiate vagally-induced bronchoconstriction to the same degree as in uninfected controls.

**5** There was no increase in baseline pulmonary inflation pressure in the infected animals over the controls. Receptors on airway smooth muscle were unchanged by viral infection since large doses of pilocarpine caused equivalent bronchoconstriction in both groups of animals. Gallamine inhibited the vagally-induced fall in heart rate equally in both groups of animals indicating that virus-induced changes in M<sub>2</sub> receptor function on pulmonary parasympathetic nerves are not part of a generalized decrease in M<sub>2</sub> receptor function.

**6** These results demonstrate that the M<sub>2</sub> muscarinic receptor-mediated inhibition of acetylcholine release from parasympathetic nerves in the lungs is decreased in animals infected with parainfluenza virus. Loss of this inhibition would result in increased release of acetylcholine from the parasympathetic nerves and may explain virus-induced airway hyperresponsiveness.

## Introduction

Viral infections of the lung exacerbate asthma in children and in adults (Frick *et al.*, 1979; Henderson *et al.*, 1979; Welliver, 1983; Little *et al.*, 1978). In normal subjects viral infection of the lung produces temporary increases in baseline airways resistance (Johanson *et al.*, 1969; Picken *et al.*, 1972; Blair *et al.*, 1976; Hall *et al.*, 1976) and increases bronchial reactivity to a variety of stimuli (Aquilina *et al.*, 1980; Empey *et al.*, 1976; Little *et al.*, 1978). These changes often persist for weeks beyond the period of clinical illness.

The mechanisms by which viruses induce airway hyperresponsiveness are poorly understood. There is no evidence that viral infection causes abnormalities in airway smooth muscle function. Contraction of airway smooth muscle *in vitro* to muscarinic agonists and histamine has been reported to be unaltered by viral infection (Buckner *et al.*, 1981; 1985; Jacoby *et al.*, 1988). *In vivo*, the bronchoconstrictor response to aerosolized acetylcholine (ACh) is the same in guinea-pigs infected with parainfluenza virus as in sham-infected animals (Dusser *et al.*, 1989).

Virus-induced hyperresponsiveness may be the result of a defect in the parasympathetic nervous system. In normal subjects, bronchoconstriction induced by exercise or by inhalation of histamine or cold air was temporarily potentiated during and immediately after respiratory viral infections (Empey *et al.*, 1976; Aquilina *et al.*, 1980). Both of these

responses were blocked by atropine indicating potentiation of a vagal reflex. A defect in the efferent limb of the parasympathetic nervous system was suggested by Buckner *et al.* (1985) who demonstrated that bronchoconstriction induced by electrical stimulation of the vagus nerves was potentiated in virus-infected guinea-pigs.

In the airways, release of ACh is under the local control of muscarinic receptors on postganglionic, parasympathetic nerves (Fryer & Maclagan, 1984; 1987a,b; Blaber *et al.*, 1985; Faulkner *et al.*, 1986). Under physiological conditions these autoreceptors inhibit ACh release, thereby limiting vagally-induced bronchoconstriction. Blockade of these receptors with selective antagonists such as gallamine potentiates vagally-mediated bronchoconstriction as much as 10 fold. Therefore, it is possible that loss of these neuronal receptors may contribute to virus-induced hyperresponsiveness. These experiments were carried out to determine whether virus infection alters the function of inhibitory muscarinic receptors on the pulmonary parasympathetic nerves.

## Methods

### Virus infection

Parainfluenza type 3 (ATCC VR-93) was grown in Rhesus monkey kidney cell monolayers in L-15 medium for one week at 34°C. Cells and medium were frozen and thawed, cleared by low-speed centrifugation, and stored in aliquots at –70°C.

Specific antigen-free guinea-pigs were anaesthetized with

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methohexitone ( $20 \text{ mg kg}^{-1}$ , i.p.). Animals in the infected group were inoculated intranasally with 1 ml virus solution that contained  $10^5 \text{ TCID}_{50} \text{ ml}^{-1}$  ( $10^5$  times the concentration required to produce infection in 50% of Rhesus monkey kidney monolayers), obtained by diluting the viral stock in Dulbecco's phosphate-buffered saline. Animals in the uninfected (control) group were inoculated intranasally with fluids obtained from virus-free Rhesus monkey kidney cells that were prepared and diluted in phosphate-buffered saline in the same way as the viral solutions. Control and infected animals were housed in separate laminar flow rooms.

#### *Virus isolation and titration*

After physiological studies were completed, the guinea-pig lungs were removed and stored at  $-70^\circ\text{C}$ . Frozen samples were thawed, weighed, and homogenized in 2 ml phosphate-buffered saline (Polytron, Brinkman, Lucerne, Switzerland). Virus was eluted from the tissue homogenate by incubation at  $34^\circ\text{C}$  for 1 h. The suspensions were centrifuged at  $400g$  for 30 min, and the supernatants were inoculated in serial 10 fold dilutions into fresh Rhesus monkey kidney cell monolayers. After one week's incubation at  $34^\circ\text{C}$ , the monolayers were washed and the medium was replaced with a 0.5% suspension of guinea-pig erythrocytes. After 1 h, the erythrocytes were washed off, and the monolayers were examined under an inverted phase-contrast microscope (Olympus) for evidence of haemadsorption (sticking of erythrocytes to the surface of cells expressing the viral haemagglutinin on their surfaces) (Shelokov *et al.*, 1958). Only data from virus-exposed guinea-pigs with proven parainfluenza infections are reported.

#### *Anaesthesia*

Guinea-pigs (Dunkin Hartley; 250–350 g) were used four days after inoculation with virus or with control media. They were anaesthetized with urethane ( $1.5 \text{ g kg}^{-1}$ ) injected intraperitoneally. This dose of urethane produces a deep anaesthesia lasting 8–10 h (Green, 1982). None of the experiments described lasted for longer than 3 h and depth of anaesthesia was monitored by observing for fluctuations in heart rate and blood pressure. Guinea-pigs were handled in accordance with the standards established by the U.S.A. Animal Welfare Acts set forth in 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.

#### *Measurement of pulmonary inflation pressure (Ppi)*

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. Both vagi were cut and the distal portions placed on shielded platinum electrodes immersed in a pool of liquid paraffin. The animal's body temperature was maintained at  $37^\circ\text{C}$  with a heating blanket.

The animals were paralysed with suxamethonium (infused at  $10 \mu\text{g kg}^{-1} \text{ min}^{-1}$ ) and ventilated with a positive pressure, constant volume animal ventilator (Harvard). Airflow was recorded as the pressure drop across a Fleisch pneumotachograph (3/0) measured with a Grass differential pressure transducer (PT5B). The airflow signal was integrated to give tidal volume. Pulmonary inflation pressure (Ppi) was measured with a Spectromed pressure transducer (DTX). All signals were displayed on a Grass polygraph.  $\text{PO}_2$  and  $\text{PCO}_2$  were measured in arterial blood samples at the beginning and end of each experiment (Corning 170 pH/blood gas analyser).

A positive pressure of 85–100 mmH<sub>2</sub>O 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 & Brodie, 1903). The sensitivity of the method was increased by a baseline sub-

tractor device (University of Maryland, Dept. of Biophysics) which cut off the basal inflation pressure allowing the increase in Ppi to be recorded at a greater amplification on a separate channel of the polygraph (Burden & Parkes, 1971). With this method, increases in pressure as small as 2–3 mmH<sub>2</sub>O could be recorded accurately. Changes in pulmonary inflation pressure reflect changes in resistance and compliance of the lungs.

Simultaneous stimulation of both vagus nerves (2–15 Hz, 0.2 ms, 5–30 V), produced a bronchoconstriction and a fall in heart rate. The nerves were stimulated regularly at 2 min intervals and the number of pulses per train were kept constant for each set of experiments. All animals were pretreated with guanethidine  $5 \text{ mg kg}^{-1}$ , i.v. This dose of guanethidine has been demonstrated to deplete noradrenaline and it produced a temporary reduction in the magnitude of the vagally-induced bronchoconstriction and bradycardia. Thirty minutes after guanethidine, when the responses to stimulation of the vagus nerves were back to pre-guanethidine values and were reproducible, cumulative dose-response curves measuring the effect of pilocarpine or gallamine on vagally-induced bronchoconstriction were performed. Doses of pilocarpine greater than  $30 \mu\text{g kg}^{-1}$  produced a transient bronchoconstriction. Therefore, the effect of these doses of pilocarpine on vagally-induced bronchoconstriction was measured after the Ppi had returned to baseline. At the end of each experiment vagally-induced bronchoconstriction and bradycardia were abolished by atropine ( $1 \text{ mg kg}^{-1}$ , i.v.) indicating that both of these responses were mediated via release of ACh onto muscarinic receptors.

#### *Drugs*

Gallamine, pilocarpine, suxamethonium, atropine, and urethane were purchased from Sigma, St. Louis, MO, U.S.A.; guanethidine was supplied by CIBA, Summit, NJ, U.S.A.; and methohexitone was purchased from Eli Lilly, Indianapolis, IN, U.S.A. All drugs were dissolved and diluted in 0.9% NaCl solution. Rhesus monkey kidney cells were purchased from Viomed, Minnetonka, MN, U.S.A.

#### *Statistics*

The effects of viral infection on dose-response curves to pilocarpine and gallamine were compared by two-way analysis of variance. The initial responses to stimulation of the vagus nerves were compared between control and infected guinea-pigs by unpaired Student's *t* tests. A *P* value less than 0.05 was considered significant.

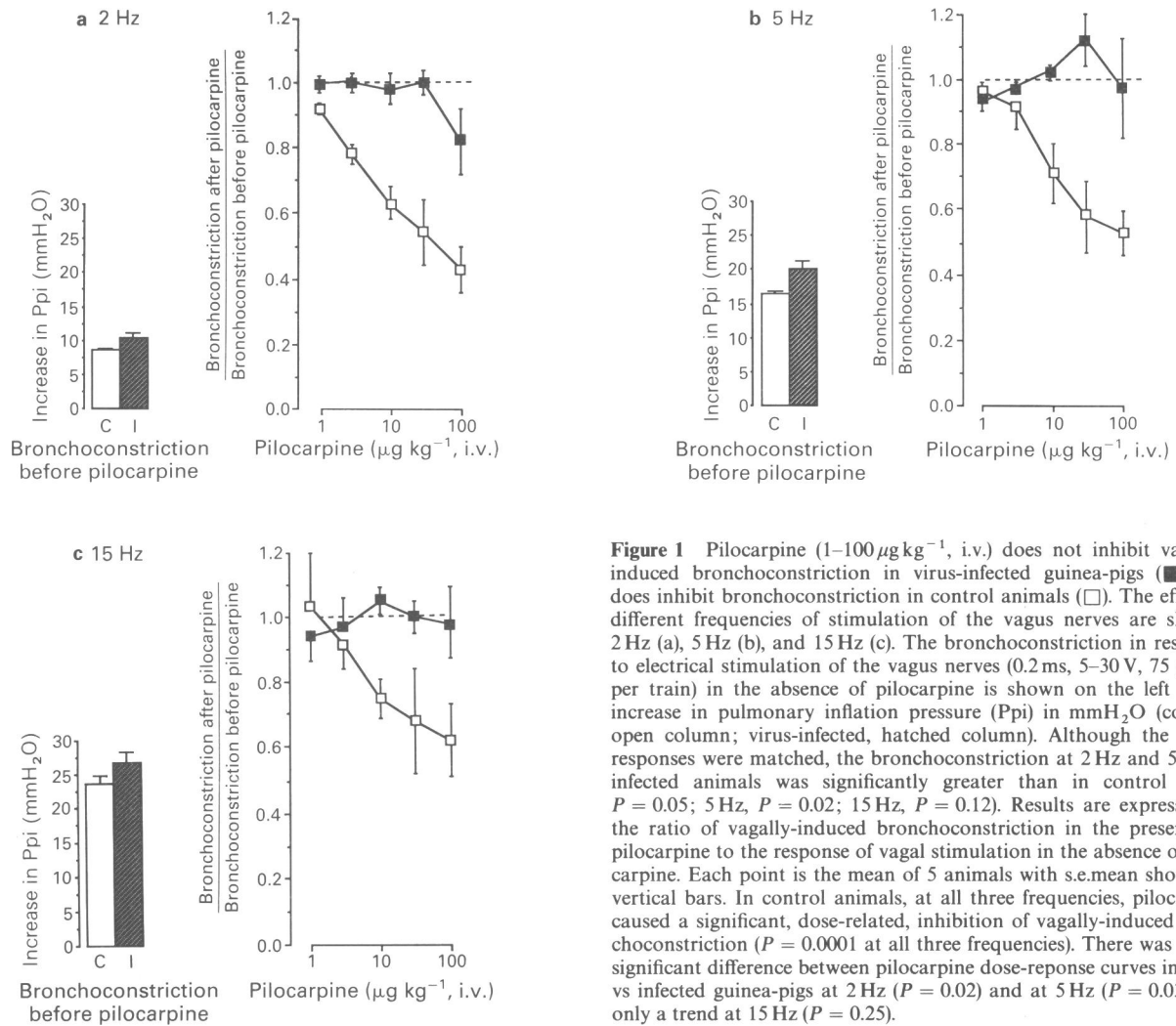
#### **Results**

All virus-exposed animals became infected with the virus. Homogenates of lungs from virus-exposed guinea-pigs contained  $10^{4.7} \pm 10^{0.22} \text{ TCID}_{50}/100 \text{ mg}$  tissue weight (geometric mean  $\pm$  s.e.mean). Lungs from control guinea-pigs contained no titratable virus.

There was no difference in baseline Ppi (85–100 mmH<sub>2</sub>O) or baseline heart rate (280–320 beats  $\text{min}^{-1}$ ) between control and virus-infected guinea-pigs. Electrical stimulation of the vagus nerves caused bronchoconstriction (measured as an increase in Ppi) and bradycardia in both groups. There were no differences between control and infected guinea-pigs in these responses (see histograms in Figures 1, 3 and 4).

In control guinea-pigs, stimulation of M<sub>2</sub> muscarinic receptors on the parasympathetic nerves by pilocarpine inhibited vagally-induced bronchoconstriction in dose-dependent fashion. The degree of inhibition was also dependent on the frequency of electrical stimulation. Pilocarpine was somewhat more effective in inhibiting bronchoconstriction elicited at 2 Hz compared with 15 Hz (Figure 1a–c, open squares).

In guinea-pigs infected with parainfluenza virus, pilocarpine was not an effective inhibitor of vagally-induced broncho-



**Figure 1** Pilocarpine (1–100 μg kg<sup>-1</sup>, i.v.) does not inhibit vagally-induced bronchoconstriction in virus-infected guinea-pigs (■) but does inhibit bronchoconstriction in control animals (□). The effect at different frequencies of stimulation of the vagus nerves are shown: 2 Hz (a), 5 Hz (b), and 15 Hz (c). The bronchoconstriction in response to electrical stimulation of the vagus nerves (0.2 ms, 5–30 V, 75 pulses per train) in the absence of pilocarpine is shown on the left as an increase in pulmonary inflation pressure (Ppi) in mmH<sub>2</sub>O (control, open column; virus-infected, hatched column). Although the initial responses were matched, the bronchoconstriction at 2 Hz and 5 Hz in infected animals was significantly greater than in control (2 Hz,  $P = 0.05$ ; 5 Hz,  $P = 0.02$ ; 15 Hz,  $P = 0.12$ ). Results are expressed as the ratio of vagally-induced bronchoconstriction in the presence of pilocarpine to the response of vagal stimulation in the absence of pilocarpine. Each point is the mean of 5 animals with s.e.mean shown by vertical bars. In control animals, at all three frequencies, pilocarpine caused a significant, dose-related, inhibition of vagally-induced bronchoconstriction ( $P = 0.0001$  at all three frequencies). There was also a significant difference between pilocarpine dose-response curves in sham vs infected guinea-pigs at 2 Hz ( $P = 0.02$ ) and at 5 Hz ( $P = 0.03$ ), but only a trend at 15 Hz ( $P = 0.25$ ).

constriction. At 2 Hz, only the highest dose of pilocarpine (100 μg kg<sup>-1</sup>) inhibited vagally-induced bronchoconstriction (Figure 1a), while at 5 and 15 Hz no dose of pilocarpine was able to inhibit vagally-induced bronchoconstriction in virus-infected guinea-pigs (Figure 1b,c). Doses greater than 100 μg kg<sup>-1</sup> pilocarpine were not used because they caused sustained bronchoconstriction via stimulation of muscarinic receptors on the airway smooth muscle.

In both control and infected guinea-pigs, pilocarpine caused a transient bronchoconstriction (at 30–100 μg kg<sup>-1</sup>) and fall in heart rate (at 1–100 μg kg<sup>-1</sup>) by stimulating muscarinic receptors on airway smooth muscle and cardiac muscle. There were no differences in these responses between the control and infected animals (Figure 2).

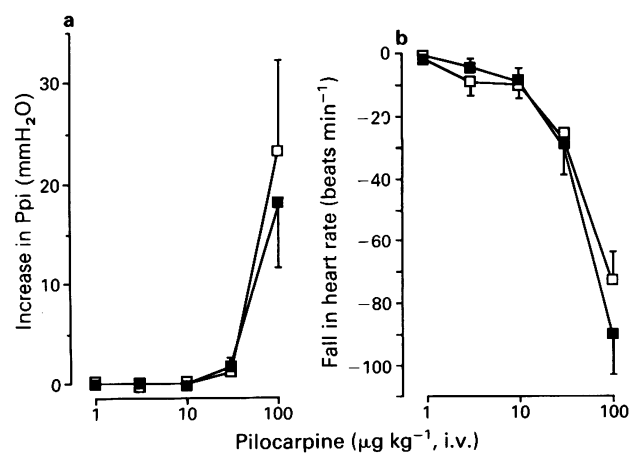
In control animals gallamine (0.1–10 mg kg<sup>-1</sup>) potentiated vagally-induced bronchoconstriction in a dose-dependent fashion. In guinea-pigs infected with parainfluenza virus this potentiation was attenuated, and the dose-response curve shifted approximately one log unit to the right (Figure 3). In the heart, gallamine inhibited vagally-induced bradycardia to the same extent in both control and virus-infected animals (Figure 4).

## Discussion

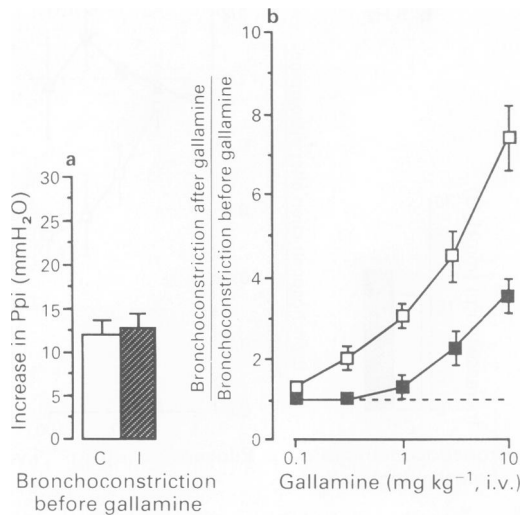
In control guinea-pigs, pilocarpine inhibited and gallamine potentiated bronchoconstriction elicited by electrical stimulation of the vagus nerves. These effects are due to stimulation (pilocarpine) and blockade (gallamine) of inhibitory M<sub>2</sub> muscarinic receptors on the pulmonary parasympathetic

nerves (Fryer & Maclagan, 1984; 1987a,b; Blaber *et al.*, 1985; Faulkner *et al.*, 1986).

Both pilocarpine-induced inhibition and gallamine-induced potentiation of vagally-mediated bronchoconstriction were markedly decreased in guinea-pigs infected with parainfluenza virus. Thus the neuronal M<sub>2</sub> receptors cannot be stimulated by exogenous agonists since pilocarpine did not inhibit

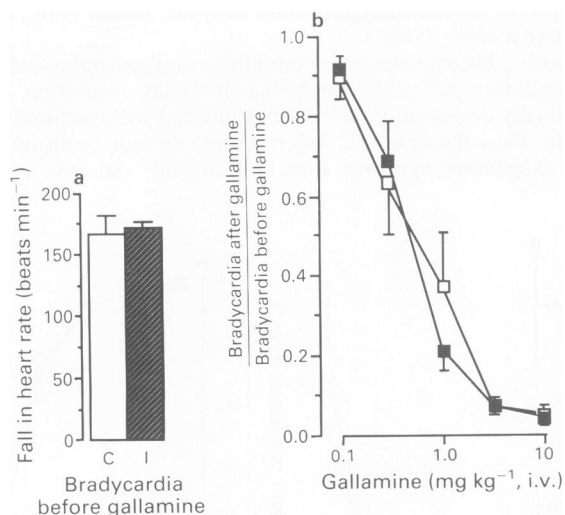


**Figure 2** Pilocarpine (1–100 μg kg<sup>-1</sup>, i.v.) causes a similar degree of bronchoconstriction (a) measured as an increase in pulmonary inflation pressure (Ppi) and bradycardia (b) measured as a fall in heart rate in control (□) and virus-infected (■) guinea-pigs. Each point is the mean of 5 animals with s.e.mean shown by vertical bars.



**Figure 3** Potentiation of vagally-induced bronchoconstriction by gallamine (0.1–10 mg kg<sup>-1</sup>, i.v.) is attenuated in guinea-pigs which are infected with parainfluenza virus (■) compared to control guinea-pigs (□) (b). The bronchoconstriction in response to electrical stimulation of the vagus nerves (0.2 ms, 15 Hz, 5–30 V, 75 pulses per train) in the absence of gallamine is shown in (a) as an increase in pulmonary inflation pressure (Ppi) in mmHg (control, open column; virus-infected, hatched column; there was no significant difference between these responses,  $P = 0.29$ ). Results are expressed as the ratio of the response to vagal stimulation in the presence of gallamine to the response to vagal stimulation in the absence of gallamine. Each point is the mean of 5 animals with s.e.mean shown by vertical bars. In control animals, gallamine caused a significant, dose-related, potentiation of vagally-induced bronchoconstriction ( $P = 0.0001$ ). There was also a significant difference between gallamine dose-response curves in sham vs infected guinea-pigs ( $P = 0.013$ ).

vagally-induced bronchoconstriction. Neither can these receptors be stimulated by endogenous ACh, since if ACh were stimulating these autoreceptors blockade by gallamine would have potentiated vagally-induced bronchoconstriction.



**Figure 4** Gallamine (0.1–10 mg kg<sup>-1</sup>, i.v.) causes a similar degree of inhibition of vagally-induced bradycardia in control (□) and virus-infected (■) guinea-pigs (b). Bradycardia elicited by electrical stimulation of the vagus nerves (0.2 ms, 15 Hz, 5–30 V, 75 pulses per train) was measured. The bradycardia in response to vagal stimulation in the absence of gallamine is shown in (a) as a fall in heart rate in beats min<sup>-1</sup> (control, open column; virus-infected, hatched column; there was no statistical difference between these responses,  $P = 0.34$ ). Results are expressed as the ratio of the response to vagal stimulation in the presence of gallamine to the response to vagal stimulation in the absence of gallamine. Each point is the mean of 5 animals with s.e.mean shown by vertical bars.

Damage to neuronal M<sub>2</sub> receptors would be expected to increase the baseline bronchoconstrictor response to vagal stimulation in virus-infected animals. Buckner *et al.* (1985) demonstrated such an increase in guinea-pigs infected with parainfluenza virus type 3. In our study, we found a great deal of variability in the response to electrical stimulation of the vagus nerves, which we attribute to variation in the isolation and preparation of the vagus nerves. A different voltage (range 5–30 V) was selected, and kept constant throughout each experiment in order that the initial bronchoconstrictor responses were similar between sham-infected and virus infected guinea-pigs. Because the voltages were varied between experiments the results of Buckner *et al.* (1985) were not directly confirmed. However, there was a trend (although not statistically significant) indicating that a given bronchoconstriction was reached with less voltage in virus-infected animals ( $11.0 \pm 2.8$  V) than in sham-infected animals ( $18.2 \pm 5.0$  V).

The function of neuronal muscarinic receptors is dependent upon the frequency at which the vagus nerve is stimulated (Fryer & Maclagan, 1984). These receptors function best, and the effects of antagonists at these receptors are most apparent, when the nerves are stimulated at higher frequencies (5–15 Hz). Conversely it is easier to demonstrate the effect of exogenous agonists when the nerve is firing at lower frequencies. We have confirmed that pilocarpine is a more effective agonist at 2 Hz in control guinea-pigs (Figure 2). In virus-infected animals we tested whether the effect of pilocarpine was inhibited at a range of frequencies because the vagus nerves in the lung fire normally at 12–15 Hz (Mitchell *et al.*, 1987).

The changes in the effects of gallamine and pilocarpine on vagally-induced bronchoconstriction cannot be accounted for by alterations in resistance since baseline Ppi was the same in both infected and control animals. Neither of these effects is related to changes in muscarinic receptors on airway smooth muscle since the pilocarpine-induced bronchoconstriction was identical in control and virus-infected animals (see Figure 2a).

Parainfluenza virus primarily infects the airway epithelium and does not usually spread to infect tissues outside the lungs. Therefore, M<sub>2</sub> receptors in other organs, such as the heart, should be unchanged by viral infection of the lungs. That virus-induced changes in M<sub>2</sub> receptors in the lung are not part of a generalized decrease in M<sub>2</sub> receptor function was demonstrated since bradycardia (mediated by M<sub>2</sub> muscarinic receptors, Hammer *et al.*, 1986) in response to both vagal stimulation (Figure 4a) and pilocarpine (Figure 2b) was not altered from control. Furthermore, gallamine was equally potent in inhibiting vagally-induced bradycardia in control and virus-infected animals (Figure 4).

Viral infection increases vagally-mediated reflex bronchoconstriction in guinea-pigs (Buckner *et al.*, 1985) and humans (Empey *et al.*, 1986; Aquilina *et al.*, 1980). The mechanism for this potentiation is unclear. Since bronchoconstriction in response to stimulation of the vagus nerves was potentiated in guinea-pigs infected with parainfluenza virus (Buckner *et al.*, 1985) it appears that at least part of this potentiation results from a defect in the efferent limb of the reflex. Damage to the inhibitory M<sub>2</sub> muscarinic receptors on the pulmonary vagus nerves, as we have demonstrated, may explain this increase in vagally-induced bronchoconstriction.

The mechanism of these changes in M<sub>2</sub> muscarinic receptor function in the airways of virus-infected guinea-pigs is unknown. We have recently shown that exposure of membrane preparations of guinea-pig lungs to parainfluenza virus *in vitro* decreases the affinity of agonists for a portion of the muscarinic receptors (Fryer *et al.*, 1990). This effect is due to viral neuraminidase, as it can be mimicked by an equivalent concentration of purified neuraminidase and blocked by a neuraminidase inhibitor. Because exposure to parainfluenza virus *in vitro* caused a similar decrease in agonist affinity for all of the muscarinic receptors in a membrane preparation of guinea-pig heart, which contains only M<sub>2</sub> receptors, it is pos-

sible that viral neuraminidase is decreasing agonist affinity selectively for M<sub>2</sub> receptors in the lung. This is consistent with the fact that M<sub>2</sub> muscarinic receptors contain a large number of sialic acid residues (Peterson *et al.*, 1986) which would be susceptible to cleavage by neuraminidase and that the sialic acid residues are involved in agonist binding to M<sub>2</sub> receptors (Gies & Landry, 1988).

M<sub>2</sub> muscarinic receptors on parasympathetic nerves in the lungs normally function to inhibit release of ACh from parasympathetic nerves in the lungs. Blockade of these autoreceptors removes the negative feedback control they provide, resulting in a potentiation of vagally-induced bronchoconstriction. The data presented here demonstrate that in

guinea-pigs infected with parainfluenza virus, inhibition of ACh release by neuronal M<sub>2</sub> muscarinic receptors in the lungs is decreased. Loss of this inhibitory control would result in potentiation of any vagally-mediated bronchoconstriction, including reflex bronchoconstriction. It is possible that virus-induced airway hyperresponsiveness is the result of viral damage to neural M<sub>2</sub> muscarinic receptors in the lungs.

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