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Inhibition of neuronal M_2 muscarinic receptor function in the lungs by extracellular nitric oxide

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1 These experiments were carried out to test whether neuronal M_2 muscarinic receptor function in the lungs is affected by nitric oxide (NO) and whether the source of the NO is epithelial or neuronal.

2 In pathogen free, anaesthetized guinea-pigs, the muscarinic agonist pilocarpine inhibited vagally induced bronchoconstriction demonstrating functional neuronal M_2 muscarinic receptors. In the presence of the NO donor, 3-morpholino-sydnonimine (SIN-1), pilocarpine no longer inhibited vagally induced bronchoconstriction. In contrast, inhibiting endogenous NO with N^G-monomethyl-L-arginine methyl ester (L-NMMA) did not affect the ability of pilocarpine to decrease vagally induced bronchoconstriction.

3 In isolated tracheas, pilocarpine inhibited contractions induced by electrical field stimulation demonstrating that neuronal M_2 muscarinic receptors function *in vitro*. As in the anaesthetized guinea-pigs, SIN-1 shifted the pilocarpine dose response curve to the right, demonstrating decreased neuronal M_2 receptor function. However, *in vitro*, L-NMMA shifted the pilocarpine dose response curve to the left, demonstrating that endogenous NO was inhibiting the ability of the M_2 receptors to decrease acetylcholine (ACh) release.

4 Both haemoglobin (Hb), which scavenges NO, and epithelial removal also shifted the pilocarpine dose response curve to the left, demonstrating that the NO inhibiting neuronal M_2 receptor function was extracellular and probably of epithelial origin.

5 In conclusion, extracellular NO appears to inhibit the ability of the M_2 receptors to decrease ACh release from the parasympathetic nerves in the lungs *in vivo* and *in vitro* in pathogen free guinea-pigs. However, while the neuronal M_2 receptors will respond to NO (from SIN-1) *in vivo*, there does not appear to be an endogenous source of NO since L-NMMA had no effect *in vivo*.
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Abbreviations: ACh, acetylcholine; D-NMMA, NG-monomethyl-D-arginine methyl ester; EFS, electrical field stimulation; Hb, haemoglobin; L-NMMA, NG-monomethyl-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; Ppi, pulmonary inflation pressure; SIN-1, 3-morpholino-sydnonimine

Introduction

Prejunctional M_2 muscarinic receptors on the parasympathetic nerves in the lungs inhibit release of acetylcholine from these nerves (Fryer & Maclagan, 1984; Fryer & Jacoby, 1993). Blockade of these neuronal M_2 muscarinic receptors with the selective muscarinic antagonist, gallamine, potentiates vagally induced bronchoconstriction. In contrast, stimulation of neuronal M_2 muscarinic receptors with the agonist, pilocarpine, inhibits vagally induced bronchoconstriction (Fryer & Maclagan, 1984). The neuronal M_2 muscarinic receptors have a strong influence on the release of acetylcholine from the parasympathetic nerves in the lungs. Loss of neuronal M_2 receptor function, as seen in animals that are sensitized and challenged with antigen, results in airway hyperreactivity (Costello *et al*., 1999).

Muscarinic receptors in the central nervous system are coupled to NO since stimulation of muscarinic receptors in rat primary cortical cultures increases cyclic GMP. This effect is blocked by inhibitors of NOS such as L-NMMA (Castoldi *et al*., 1993). Although the subtype of muscarinic receptors coupled to NO in the brain were not identified, it has been demonstrated that M_2 receptors are linked to neuronal NOS in cultured cells (Wang *et al*., 1997). In the heart, stimulation of M_2 muscarinic receptors increases NO and decreases heart rate (Yamamoto *et al*., 1998). L-NMMA inhibited the M_2 mediated fall in heart rate and increase in NO, suggesting that production of NO following M_2 receptor stimulation causes bradycardia. M_2 muscarinic receptor function appears then to involve NO.

NOS is present in the epithelium, endothelium and nerves within the lungs (Kobzik *et al*., 1993; Rengasamy *et al*., 1994; Guo *et al*., 1995; Sherman *et al* 1999). It has been suggested that epithelial NO may diffuse to the airway smooth muscle (Rengasamy *et al*., 1994) where it may contribute to relaxation of the airways (Gaston *et al*., 1994; Hirasaki *et al*., 1996). Epithelial NO may then also reach the neuronal M_2 muscarinic receptors within airway smooth muscle. These experiments were carried out to determine whether the function of the M_2 muscarinic receptors on parasympathetic nerves in the lungs is affected by NO, and whether the NO was from the epithelium. The function of M_2 receptors was tested both *in vitro* in the absence and presence of airway epithelium, and *in vivo*. These experiments were carried out using the agonist pilocarpine or antagonist gallamine in the presence of the NOS inhibitor L-NMMA and in the presence of the NO donor SIN-1.

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Methods

Animals

Dunkin-Hartley guinea-pigs (350–400 g; supplied by Hilltop Animal Farms, Scottsdale, PA, U.S.A.), bred specific pathogen-free, and maintained in barrier housing were used. All guinea-pigs were shipped in filtered crates. Upon arrival, they were housed in wire bottom cages inside laminar flow hoods. Masks and gloves were worn by all personnel entering the room or handling the guinea-pigs. 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.

Anaesthesia and measurement of pulmonary inflation pressure (Ppi)

Guinea-pigs were anaesthetized with urethane (1.5 g kg^{-1} i.p.). This dose of urethane produces a deep anaesthesia that lasts 8–10 h (Green, 1982) although the experiments described here were completed within 3 h.

The right carotid artery was cannulated to measure blood pressure with a transducer (DTX, Spectramed, Oxnard, CA, U.S.A.). Heart rate was derived electronically from the pressure signal. The jugular veins were cannulated for the administration of drugs. Both vagus nerves were cut, and the distal ends were placed on platinum electrodes and bathed in a pool of mineral oil. Animals were tracheostomized, ventilated with a positive-pressure, constant-volume rodent ventilator (Harvard Apparatus, South Natick, MA, U.S.A.) at a tidal volume of 0.01 ml g^{-1} body weight and 100 breaths min^{-1}; they were paralyzed with a constant infusion of suxamethonium (10 μg kg^{-1} i.v.).

Ppi was measured at the trachea by using a pressure transducer (DTX, Spectramed). A positive pressure of 100–120 mmH_2O was needed to adequately ventilate the animals. Signals were recorded on a separate channel at a higher sensitivity. This method provides a way to measure accurately increases in Ppi as small as 2–3 mmH_2O above the baseline.

Measurement of vagally induced bronchoconstriction

All animals were pretreated with guanethidine (5 mg kg^{-1} i.v.) to deplete noradrenaline at least 25 min before the start of the experiment (Blaber & Fryer, 1985). Electrical stimulation of both vagus nerves (see below) produced bronchoconstriction (measured as an increase in Ppi) and bradycardia; both results were due to release of acetylcholine onto muscarinic receptors, because they were abolished by atropine (1 mg kg^{-1} i.v.).

Measurement of neuronal M_2 muscarinic receptor function

Pilocarpine is a muscarinic agonist with some selectivity for neuronal over postjunctional receptors (Langley, 1878). Conversely, gallamine is an antagonist that is selective for M_2 muscarinic receptors (Riker & Wescoe, 1951). Neuronal M_2 muscarinic receptor function was measured by the ability of pilocarpine to inhibit, and of gallamine to potentiate vagally induced bronchoconstriction in a dose-related manner (Fryer & Maclagan, 1984). The effects of pilocarpine and gallamine are frequency dependent. The effect of pilocarpine is greater at 2 Hz than at higher frequencies; while the effect of gallamine is greater at higher frequencies (Fryer & Maclagan, 1984). Thus, all experiments using pilocarpine were carried out at 2 Hz; and those with gallamine were carried out at 15 Hz. Both vagus nerves were electrically stimulated (2 Hz, 0.2 ms, for 22 s; or 15 Hz, 0.2 ms, for 3 s) at 1 min intervals. In the absence of muscarinic agonist or antagonist, the initial voltage was chosen within the range of 5–15 V to give an increase in Ppi of 15–25 mmH_2O for pilocarpine and 10–12 mmH_2O for gallamine experiments. Once the voltage was determined it was not varied within the experiment.

Once the vagally induced bronchoconstrictions were stable and consistent, increasing doses of pilocarpine (0.1–100 μg kg^{-1} i.v.) or gallamine (0.1–10 mg kg^{-1} i.v.) were administered. The effect on vagally induced bronchoconstriction was measured as the ratio of bronchoconstriction in the presence of pilocarpine or gallamine to bronchoconstriction in their absence.

Administration of nitric oxide synthase inhibitor and NO donor

Some animals were treated with the NOS inhibitor N^G-monomethyl-L-arginine methyl ester, L-NMMA (20 mg kg^{-1} i.v.) or the inactive enantiomer D-NMMA (20 mg kg^{-1} i.v.) 30 min prior to vagally induced bronchoconstriction. Other animals were treated with an infusion of the NO donor, SIN-1 (20 μg kg^{-1} min^{-1} i.v.; Friggi *et al*., 1989).

Response of isolated tracheas to electrical field stimulation

Pathogen free guinea-pigs were killed with an overdose of urethane (4.0 g kg^{-1} i.p.). Their tracheas were removed and placed in Krebs-Henseleit solution of the following composition (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25.0 and dextrose 5.55 containing propranolol (10^{-6} M) to block the effects of sympathetic nerve stimulation. The Krebs-Henseleit solution was bubbled with 5% CO_2 and 95% O_2, pH 7.4.

The tracheas were cut transversely into segments consisting of 3–5 cartilaginous rings. Each ring was mounted vertically under isometric tension in a 5 ml water jacketed organ bath kept at 37°C. Electrical field stimulation of the smooth muscle (10 Hz, 2.0 ms pulse duration, 100 V, 15 s pulse train, at 1-min intervals) produced contraction of the tracheal rings, which was reversible upon cessation of the stimulation. Electrical field stimulation-induced contractions of the isolated tracheas in the absence and then in the presence of increasing concentrations of pilocarpine (10^{-11}–10^{-3} M; added cumulatively at 5 min intervals) were measured. Data are expressed as the ratio of the contraction in the presence of pilocarpine to the contraction in the absence of pilocarpine. At the end of each experiment, atropine (10^{-4} M) was administered to the muscle baths to ensure that the contractions in response to transmural stimulation were mediated *via* muscarinic receptors.

Some tissues were treated with the NOS inhibitor, N^G-monomethyl-L-arginine methyl ester (L-NMMA, 10^{-5} M). Other tissues were treated with the NO donor 3-morpholino-sydnonimine (SIN-1; 10^{-5} M), or with haemoglobin (10^{-5} M), a scavenger of extracellular NO (Elgavish *et al*., 1996). In some tissues airway epithelium was removed by passing a rolled kimwipe through the tracheal lumen three times prior to segmenting the trachea into rings. Epithelial removal was confirmed by histology.

Drugs

The drugs used in these experiments were: urethane, guanethidine, suxamethonium, pilocarpine, gallamine, propranolol, bovine haemoglobin, N^G-monomethyl-L-arginine methyl ester (L-NMMA), 3-morpholino-sydnonimine (SIN-1), and atropine, all purchased from Sigma Chemical Co (St Louis, MO, U.S.A.). All drugs were dissolved and diluted in 0.9% NaCl.

Statistics

All data are expressed as means ± s.e.mean. The effects of pilocarpine and gallamine on vagally induced bronchoconstriction or electrical field stimulated contraction were analysed by using two-way ANOVAs for repeated measures. Baseline heart rates, blood pressures, Ppi, changes in Ppi (before pilocarpine administration), and voltages used were analysed by ANOVA (Statview 4.5, Abacus Concepts, Berkley, CA, U.S.A.). A P value $\leqslant 0.05$ was considered significant.

Results

In vivo, baseline Ppi varied from 82 ± 10.4 mmH_2O in controls to 96.7 ± 5.56 mmH_2O post L-NMMA, 93.0 ± 9.0 mmH_2O post D-NMMA and 106.7 ± 6.7 mmH_2O post SIN-1. Their values are not significantly different from each other. Baseline heart rate varied from 281 ± 8.8 beats min^{-1} in controls to 298.3 ± 25.9 beats min^{-1} post L-NMMA, 266.0 ± 23.0 beats min^{-1} post D-NMMA and 366.3 ± 97.8 mmH_2O post SIN-1. Their values are not significantly different from each other. Baseline blood pressure varied from 62 ± 3.5 mmHg (systolic) and 33 ± 2.5 mmHg (diastolic) in controls to 53.7 ± 6.9; 84.0 ± 6.7 mmHg (systolic; diastolic) post L-NMMA, and 37.3 ± 5.3; 21.3 ± 4.4 mmHg (systolic; diastolic) post SIN-1. Both systolic and diastolic blood pressure were significantly decreased by SIN-1 compared to control and L-NMMA treated guinea-pigs.

Simultaneous electrical stimulation of both vagus nerves resulted in bronchoconstriction (2 Hz, 0.2 ms pulse duration, 22-s stimulus train, at 1-min intervals). In control animals, pilocarpine (0.1 – 100 $\mu g\ kg^{-1}$ i.v.) inhibited vagally-induced bronchoconstriction in a dose-dependent fashion by stimulating inhibitory M_2 muscarinic receptors on the pulmonary parasympathetic nerves (Figure 1, open squares). In contrast, inhibition of vagally induced bronchoconstriction by pilocarpine was significantly attenuated in guinea-pigs infused with SIN-1 (20 $\mu g\ kg^{-1}\ min^{-1}$) (Figure 1, closed squares).

There was no difference in the pilocarpine dose response curve for control animals (Figure 2, open circles) versus those treated with the NO synthase inhibitor, L-NMMA (20 $mg\ kg^{-1}$) (Figure 2, closed circles).

In animals treated with 20 $mg\ kg^{-1}$ of the inactive enantiomer, D-NMMA, gallamine (0.1 – 10 $mg\ kg^{-1}$) potentiated vagally-induced bronchoconstriction dose-dependently by blocking inhibitory M_2 muscarinic receptors on the pulmonary parasympathetic nerves (Figure 3, open circles). In the presence of L-NMMA the dose response curve to gallamine was not different from that in the presence of D-NMMA (Figure 3, closed circles).

In vitro, electrical field stimulation of tracheal rings caused contraction of the airway smooth muscle. At the end of each experiment, contractions to electrical field stimulation were abolished by atropine (10^{-4} M), indicating that these responses were mediated *via* acetylcholine release onto muscarinic receptors.

Pilocarpine inhibited field stimulation-induced contractions of the isolated tracheas in a dose-dependent manner (10^{-11} – 10^{-3} M; Figure 4, open squares). In the presence of SIN-1, the

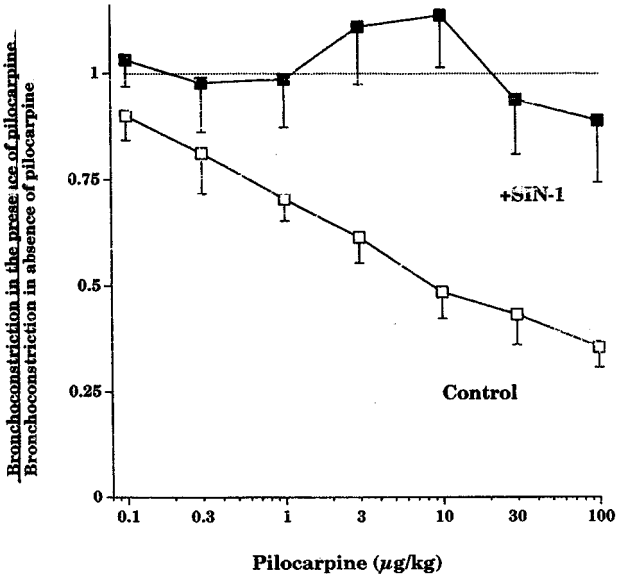

Figure 1 The inhibition of vagally-induced bronchoconstriction by pilocarpine is significantly decreased by SIN-1 *in vivo*. Electrical stimulation of the vagus nerves (2 Hz, 0.2 ms, 5–15 V, 44 pulses $train^{-1}$) causes bronchoconstriction measured as a rise in pulmonary inflation pressure in mmH_2O (in the absence of pilocarpine vagally induced bronchoconstriction, in mmH_2O, was 21.7 ± 1.4 in controls; and 18.9 ± 4.2 in SIN-1 treated guinea-pigs). Pilocarpine inhibits vagally induced bronchoconstriction in control animals, but not in animals infused with SIN-1 (20 $\mu g\ kg^{-1}\ min^{-1}$ i.v., $P = 0.02$) Each point is the mean ± s.e.mean; $n = 5$ animals.

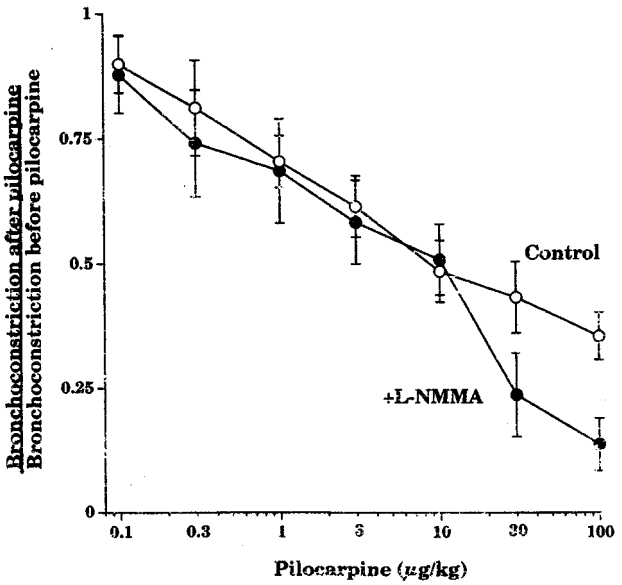

Figure 2 The inhibition of vagally-induced bronchoconstriction by pilocarpine is not affected by L-NMMA *in vivo*. Data was obtained as in Figure 1; controls are the same as in Figure 1. In the absence of pilocarpine, vagally induced bronchoconstriction in L-NMMA treated guinea-pigs was 25.1 ± 2.0 mmH_2O. Pilocarpine inhibits vagally induced bronchoconstriction both in the absence and presence of L-NMMA (20 $mg\ kg^{-1}$ i.v.). Each point is the mean ± s.e.mean; $n = 5$ animals.

dose response curve to pilocarpine was shifted significantly to the right (Figure 4, closed squares). In contrast, pretreatment of muscle baths with L-NMMA, shifts the dose response to pilocarpine significantly to the left (Figure 5, closed circles) relative to controls (Figure 5, open circles). Haemoglobin, an extracellular scavenger of NO, also caused a significant leftward shift of the dose response curve to pilocarpine (Figure 6, closed diamonds) relative to controls (Figure 6, open diamonds). Similarly, removal of the epithelial layer from the lumen of the trachea caused the dose response curve to pilocarpine to shift significantly to the left (Figure 7, closed triangles) relative to controls (Figure 7, open triangles). When L-NMMA was administered to tissues with the epithelium removed, there was no further shift in the dose response curve to pilocarpine than with either treatment alone (Figure 7, closed circles).

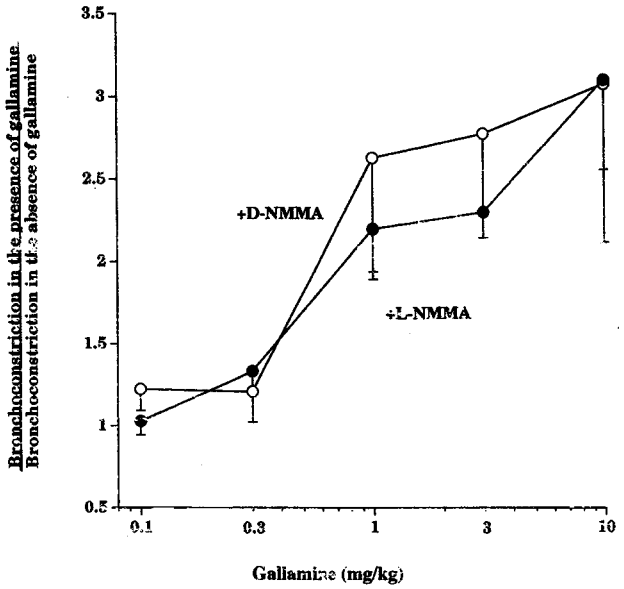

Figure 3 Gallamine potentiates vagally-induced bronchoconstriction in the presence of L-NMMA (20 mg kg^{-1} i.v.) and D-NMMA (20 mg kg^{-1} i.v.) in pathogen-free guinea-pigs *in vivo*. Electrical stimulation of the vagus nerves (15 Hz, 0.2 ms, 5–15 V, 45 pulses $train^{-1}$) causes bronchoconstriction measured as a rise in pulmonary inflation pressure in mmH_2O. In the absence of gallamine vagally induced bronchoconstriction, in mmH_2O, was 23.0±8 in D-NMMA and 23.0±0.9 in L-NMMA treated guinea-pigs. There was no significant difference between the potentiation of vagally induced bronchoconstriction by gallamine in the L- and D-NMMA treated guinea-pigs. Each point is the mean±s.e.mean; $n=5$ animals.

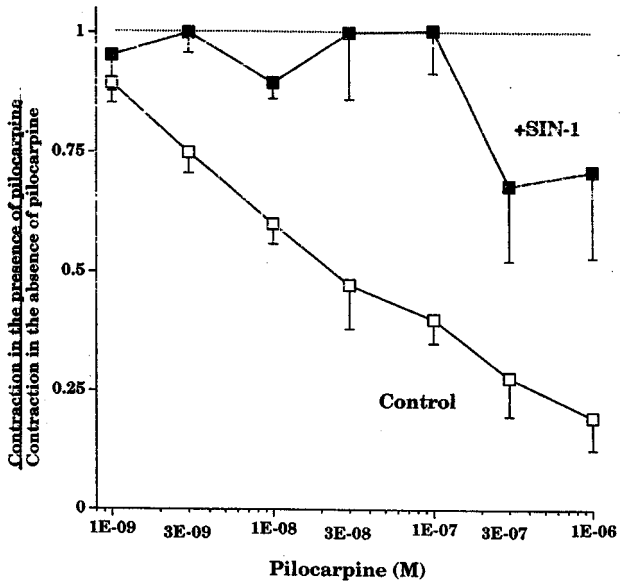

Figure 4 Electrical field stimulation (EFS) (10 Hz, 2.0 ms duration, 100 V, for 15 s, at 1-min intervals) of isolated trachea causes contraction of airway smooth muscle *in vitro*. Pilocarpine inhibits the contraction induced by EFS in control tissues. The pilocarpine dose response curve was shifted significantly to the right in the presence of SIN-1 ($P=0.004$). Each point is the mean±s.e.mean of tissue from five animals.

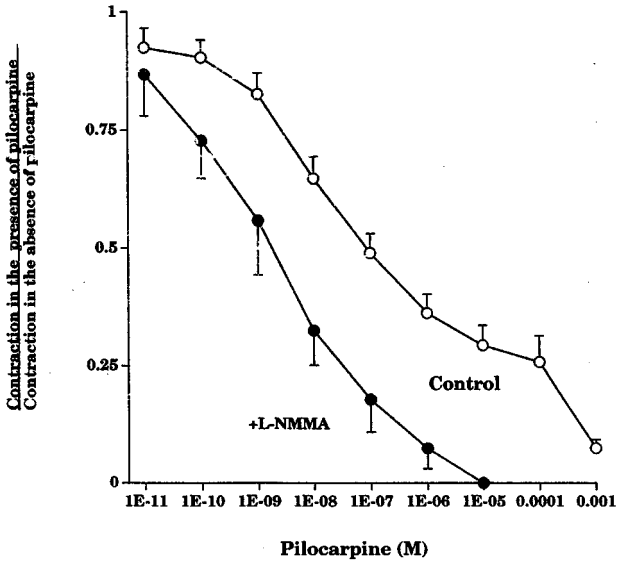

Figure 5 Electrical field stimulation of isolated trachea causes contraction of airway smooth muscle *in vitro*. Data was obtained as in Figure 4; control data is the same as in Figure 4. The dose response curve to pilocarpine was shifted significantly to the left in the presence of L-NMMA (10^{-5} M; $P=0.007$). Each point is the mean±s.e.mean of tissue from eight animals.

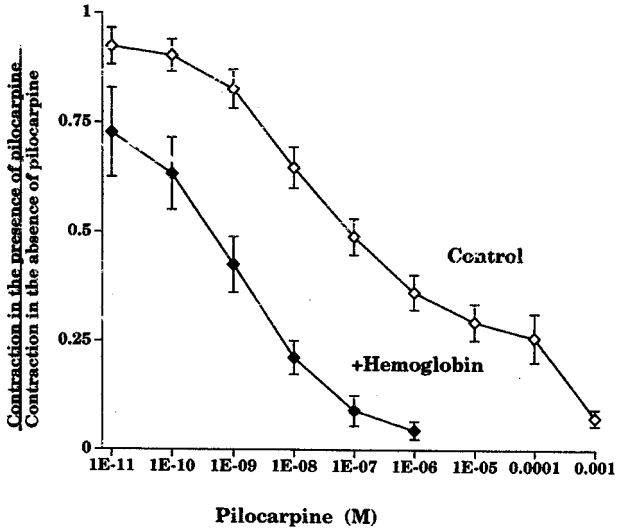

Figure 6 Electrical field stimulation of isolated trachea causes contraction of airway smooth muscle *in vitro*. Data was obtained as in Figure 4; control data is the same as in Figure 4. The dose response curve to pilocarpine was shifted significantly to the left in the presence of haemoglobin (10^{-5} M; $P<0.0001$). Each point is the mean±s.e.mean of tissue from seven animals.

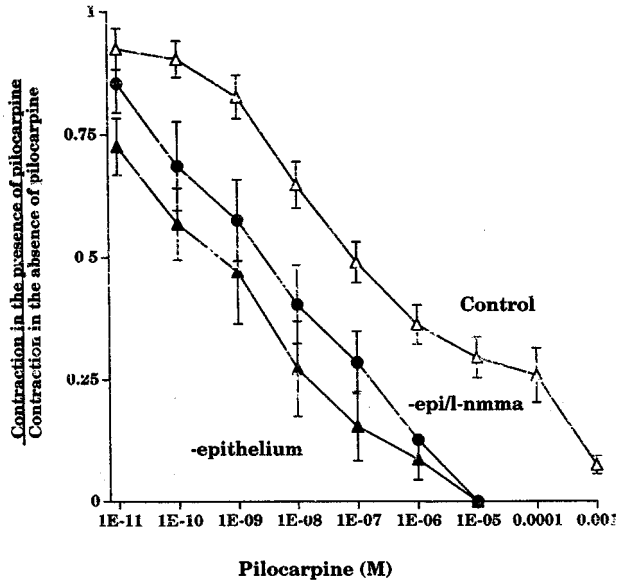

Figure 7 Electrical field stimulation of isolated trachea causes contraction of airway smooth muscle *in vitro*. Data was obtained as in Figure 4; control data is the same as in Figure 4. The dose response curve to pilocarpine was shifted significantly to the left in tissues with the epithelium removed ($P=0.0001$). Addition of L-NMMA (10^{-5} M), to tissues with epithelium removed ($P=0.0006$) did not significantly shift the dose response curve to pilocarpine further than epithelial removal alone. Each point is the mean ± s.e.mean of tissue from six animals.

Discussion

In the lungs neuronal M_2 muscarinic receptors function to inhibit release of acetylcholine from the parasympathetic nerves (Fryer & Maclagan, 1984). Blockade of these receptors potentiates release of acetylcholine leading to a significant potentiation of vagally-induced bronchoconstriction. Therefore neuronal M_2 muscarinic receptors have a marked inhibitory effect on neurotransmitter release from the parasympathetic nerves in the lungs.

In pathogen free guinea-pigs, *in vivo*, the muscarinic agonist pilocarpine, inhibited vagally induced bronchoconstriction in a dose related manner, demonstrating that the neuronal M_2 receptors were functioning to inhibit release of acetylcholine. Blockade of NOS, by administration of L-NMMA, had no effect on the pilocarpine dose response curve (Figure 2). The lack of effect of L-NMMA was not dose related since this dose has been shown to inhibit NOS in guinea-pigs (Yarkony & Fryer, 1994). Thus, the neuronal M_2 muscarinic receptors did not require NO to inhibit release of acetylcholine.

Conversely, the function of the neuronal M_2 receptors was blocked by NO since in the presence of SIN-1, pilocarpine no longer inhibited vagally induced bronchoconstriction *in vivo* (Figure 1). It might be expected that blockade of neuronal M_2 muscarinic receptor function by addition of SIN-1 should increase vagally induced bronchoconstriction; it did not. NO has been reported to inhibit ACh-induced bronchoconstriction at the level of the smooth muscle (Ward *et al*., 1993). Thus, the opposing effects of NO, increasing release of ACh from the nerves (by inhibiting M_2 receptor function) while opposing ACh induced bronchoconstriction may account for the lack of effect of SIN-1 on vagally induced bronchoconstriction *in vivo*.

The ability of NO to inhibit the function of the neuronal M_2 receptors *in vivo* was confirmed *in vitro* (Figure 4). Pilocarpine inhibited electrical field stimulation induced contractions of guinea-pig tracheal rings in control, but not in SIN-1 treated tissues. Thus, the function of the neuronal M_2 muscarinic receptors is inhibited by NO both *in vivo* and *in vitro*.

In vitro, inhibition of NO synthase by L-NMMA, shifted the dose response curve to pilocarpine to the left (Figure 5), further confirming that NO inhibits the function of the neuronal M_2 muscarinic receptors. However, *in vivo*, addition of L-NMMA did not affect the dose response curve to pilocarpine (Figure 2). Thus, while exogenous NO (derived from the NO donor SIN-1) can inhibit the function of the M_2 receptors *in vivo*, the lack of effect of L-NMMA would suggest that there is no significant, endogenous, source of NO around the nerves. NOS is present in epithelial cells as well as in the nerves in the lungs (Kobzik *et al*., 1993; Guo *et al*., 1995; Sherman *et al*., 1999). However, it appears that in pathogen free guinea-pigs, any NO produced in these tissues does not affect neuronal M_2 muscarinic receptor function *in vivo*.

In vitro, haemoglobin, an extracellular scavenger for NO shifted the dose response curve to pilocarpine to the left. This confirmed that the source of NO affecting neuronal M_2 receptor function was not intracellular. Although NOS has been identified within the parasympathetic nerves (Kobzik *et al*., 1993), the effect of haemoglobin was identical to the effect of L-NMMA, thus, all the NO inhibiting neuronal M_2 function *in vitro* was derived from extracellular, i.e. non-neuronal, sources.

Epithelial cells are known to be a source of NO (Guo *et al*., 1995; Sherman *et al*., 1999). Removal of the epithelial cells shifted the pilocarpine dose response curve to the left identically to the effects of both L-NMMA and haemoglobin. Addition of L-NMMA to epithelium removed tissues did not further shift the pilocarpine dose response curve. These results suggest that there was only one source of NO in these tissues and that this source was the airway epithelium.

In vivo, the airway epithelium is intact and forms a barrier in the airways. It is possible that NO is only secreted from the lumenal side of the epithelial cells, and thus does not normally reach the parasympathetic nerves. Cutting the trachea in preparation for *in vitro* experiments opens the epithelium, allowing NO in the medium to reach the M_2 receptors on the parasympathetic nerves. Thus, although neuronal M_2 muscarinic receptors respond to NO *in vivo*, as demonstrated by the inhibitory effect of the NO donor, SIN-1, there is no significant endogenous source of NO *in vivo*. Thus, the data demonstrating an effect of NO on M_2 receptor function in pathogen free guinea-pigs may not be physiologically relevant.

It is possible however that the neuronal M_2 receptors may be inhibited by NO derived from other non-neuronal sources *in vivo*. Inflammatory cells are known to secret NO, and exhaled NO increases with the presence of inflammation in the lungs (Gaston *et al*., 1994). The function of the neuronal M_2 muscarinic receptors is inhibited in animals exposed to ozone, infected with virus or challenged by inhaled antigen (Fryer & Jacoby, 1991; Fryer & Wills-Karp, 1991; Schultheis *et al*., 1994). All of these models are associated with inflammation of the airways. Eosinophils are found in association with the airway nerves in antigen challenged guinea-pigs, where they inhibit neuronal M_2 receptor function *via* secretion of major basic protein (Costello *et al*., 1997; Evans *et al*., 1997), an endogenous M_2 antagonist (Jacoby *et al*., 1993). Ozone exposure and viral infection are associated with an increase in neutrophils and macrophages rather than eosinophils (Yost *et al*., 1999; Adamko *et al*., 1999). Macrophages have been shown as a significant source of NO (Jorens *et al*., 1991; Tayeh & Marletta, 1989). Whether NO derived from inflammatory cells inhibits the function of neuronal M_2 receptors in these disease models remains to be tested.

It has been suggested that inhibitors of NO potentiate release of ACh from guinea-pig parasympathetic nerves *in vitro* (Belvisi *et al*., 1991). However, this is in contrast to other data demonstrating that NOS inhibitors do not alter ACh release (Brave *et al*., 1991) and to our own data demonstrating that NO inhibits the function of the neuronal M_2 muscarinic receptors, and thus would increase release of ACh. There may be several explanations for these varied findings. Indomethacin was not used in our experiments since it has been demonstrated that cyclo-oxygenase and NOS may influence each other (Tetsuka *et al*., 1994). Indomethacin was used in the other papers. In addition, the use of L-N^G-nitro-arginine methyl ester (L-NAME) to inhibit NOS is controversial since it has been suggested that L-NAME is an antagonist for M_2 muscarinic receptors (Buxton *et al*., 1993). Thus, the potentiation of parasympathetic-induced contractions of guinea-pig trachea *in vitro* by L-NAME may have been due to blockade of the neuronal M_2 receptors rather than to decreased NO release.

We have demonstrated that NO actually decreases the function of the neuronal M_2 receptors *in vitro* and *in vivo* in pathogen free guinea-pigs. In addition, neuronal M_2 muscarinic receptors do not inhibit ACh release *via* NO *in vivo* in pathogen free guinea-pigs. Pretreatment with L-NMMA had no effect on either the pilocarpine or the gallamine dose response curves. If the M_2 receptors needed NO to inhibit ACh release, L-NMMA would have blocked pilocarpine's dose-dependent inhibition of vagally induced bronchoconstriction or gallamine's dose-dependent potentiation of vagally induced bronchoconstriction.

In conclusion, these results demonstrate that M_2 muscarinic receptors do not appear to require generation of NO in order to inhibit acetylcholine release. In contrast, NO appears to inhibit the function of the M_2 receptors, through some undefined mechanism. The physiological relevance of this effect is not important in pathogen free guinea-pigs, since there appears to be no significant, endogenous, local source of NO, but may be important in pathological situations, when NO generation by inflammatory cells may affect neuronal M_2 muscarinic receptor function.

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