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Functional characterization of muscarinic receptors in murine airways

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1 The effects of muscarinic receptor antagonists considered to be selective for M_1 receptors (pirenzepine; PZ), M_2 receptors (AFDX-116), and for M_3 receptors (4-diphenyl acetoxy N-methyl-piperidine (4-DAMP)) were used to investigate the existence of muscarinic receptor subtypes in murine airways. Atropine was used as a nonselective antagonist. The effects of these antagonists were studied upon tracheal contractions induced either by EFS (electric field stimulation) or by application of an exogenous cholinoceptor agonist (arecoline).

2 The muscarinic receptor antagonists tested inhibited arecoline-induced tracheal contractions with the following rank order of potency: 4-DAMP = atropine > pirenzepine = AFDX-116. The rank order of potency of the muscarinic antagonists used in inhibiting EFS-induced tracheal contractions was: 4-DAMP = atropine > PZ > AFDX-116. The pA_2 values for these antagonists were similar when compared to the pA_2 values determined in guinea-pig and bovine airway smooth muscle.

3 In addition to *in vitro* studies, the effects of inhalation of the different muscarinic antagonists on lung function parameters *in vivo* were investigated. Inhalation of 4-DAMP induced a decrease in airway resistance and an increase in lung compliance. In contrast, inhalation of AFDX-116 induced an increase in airway resistance and almost no change in lung compliance. Apart from some minor effects of atropine on airway resistance, atropine, PZ, and pilocarpine failed to induce changes in lung mechanics as determined by *in vivo* lung function measurements.

4 The results provide evidence for the existence of M_3 receptors on murine tracheae that are involved in the contraction of tracheal smooth muscle. This is in agreement with other animal species such as the guinea-pig and bovine. *In vivo* experiments also demonstrated that in the mouse, M_3 receptors play an important role in bronchial smooth muscle contraction and thus in bronchoconstriction. Interestingly we have also demonstrated that M_2 receptors can play a role in bronchodilatation. Inhalation of an M_2 receptor antagonist induced an increase in airway resistance whereas inhalation of an M_3 receptor antagonist induced a decrease in airway resistance. It is therefore likely that an M_3/M_2 receptor balance plays an important role in the regulation of airway function.

Keywords: Muscarinic receptors; murine airways; trachea

Introduction

The parasympathetic nervous system forms the predominant neural pathway in the airways and plays an important role in airway obstruction (Nadel, 1980; Partanen *et al.*, 1982; Sheppard *et al.*, 1983; Barnes, 1984; 1986; 1987; Nadel & Barnes, 1984; De Kock & Brandt, 1985). Receptors of this system can be divided into several subtypes. At the beginning of this century it was demonstrated that the effects of acetycholine could be divided into an action at 2 subtypes of receptors: those stimulated by nicotine and those stimulated by muscarine (Dale, 1914). These 2 different receptor subtypes formed the first division in the receptors of the parasympathetic nervous system. Latterly the existence of at least three functional muscarinic receptors has been postulated (Birdsall & Hulme, 1983; Eglen & Whiting, 1986; Doods *et al.*, 1987). However more recently, the discovery of more agonists and antagonists that show preferential selectivity for muscarinic receptors in different tissues, receptor isolation, and cloning techniques have provided evidence for the existence of 5 subtypes of muscarinic receptors (m_1–m_5) (Kerlavage *et al.*, 1987).

It is possible to differentiate muscarinic receptors for which the antagonist pirenzepine (PZ) has a high affinity (denoted as M_1 receptors), those for which AFDX-116, gallamine, and methoctramine have a high affinity (denoted as M_2 receptors), and those for which 4-diphenyl acetoxy N-methyl piperidine (4-DAMP) and hexahydrosiladiphenidol have a high affinity (denoted as M_3 receptors) (Birdsall & Hulme, 1983; Eglen & Whiting, 1986; Doods *et al.*, 1987). M_1 receptors are present in parasympathetic ganglia where they appear to facilitate ganglionic neurotransmission. M_2 receptors seem to be located presynaptically on cholinergic nerve endings where they function as autoreceptors, inhibiting acetylcholine release, and M_3 receptors appear to be located on effector cells of smooth muscle and mucus secreting glands (Minette & Barnes, 1990).

Previously, we described a method to measure tracheal smooth muscle responses in mice (Garssen *et al.*, 1990). In that study the effect of *in vitro* sympathetic and parasympathetic stimulation was studied. Muscarinic receptor subtypes were not analysed. This paper describes studies in murine isolated tracheae in which the effect of a range of selective muscarinic antagonists on contraction induced either by a cholinoceptor agonist or electrical field stimulation (EFS) were investigated. These studies were carried out in order to determine the muscarinic receptor subtypes mediating and/or modulating contractile responses in this tissue. In addition, the effects of inhalation of the antagonists used on lung function parameters such as airway resistance and lung compliance were investigated *in vivo*.

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Methods

Animals

SPF BALB/c mice (male), 5–8 weeks of age, weighing 20–25 g were obtained from the breeding colony at the National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands. After delivery, the animals were rested for at least 1 week before use.

Isometric measurement of bronchial reactivity

Mice were killed by intraperitoneal (i.p.) injection of 0.25 ml nembutal (pentobarbitone sodium 60 mg ml^{-1}, Abbott Laboratories, North Chicago, IL, U.S.A.). The tracheae, resected *in toto*, were transferred to a Petri dish containing a modified oxygenated Krebs bicarbonate solution (composition mmol l^{-1}: NaCl 118.1, KCl 4.7, $CaCl_2.6H_2O$ 2.5, $MgCl_2.6H_2O$ 0.5, $NaHCO_3$ 25.0, $NaH_2PO_4.H_2O$ 1.0 and glucose 11.1). The tracheae were prepared free of excess tissue by use of a binocular preparation microscope and pieces of approximately 6 mm length (9 trachea rings just beneath the larynx) were taken. The weights of the tracheal preparations were similar. Thus, in each experiment similar tracheal sections were used (Garssen *et al.*, 1990).

The tracheae were mounted directly on two supports in an organ bath, one of which was connected to an isometric transducer and the other to a plastic holder. This procedure did not lead to tissue damage, as determined histologically. The organ baths were filled with 20 ml of Krebs solution. The solution was aerated continuously with a mixture of oxygen (95%) and carbon dioxide (5%). Temperature was maintained at 37°C with a constant temperature circulating unit (Thermomix 1460; Braun Melsungen, Germany).

Isometric measurements were made with a force displacement transducer (isometric transducer, Harvard Bioscience, Boston, U.S.A.) and a two-channel recorder (Servogor type SE-120) and were expressed as changes in force measured in grams. Optimal basic force for the mouse trachea was 1 g (Hooker *et al.*, 1977; Bartell & Busse, 1980; Garssen *et al.*, 1990). The tracheae were allowed to equilibrate for at least 1 h before drug effects were elicited. During the equilibration the bath fluid was exchanged every 15 min. Drugs were prepared in Krebs solution and kept on ice for the duration of the experiment.

Effect of muscarinic antagonists on arecoline-induced smooth muscle contraction

Cumulative contractile concentration-response curves were determined for arecoline. After the determination of this curve the bath fluid was exchanged 4 times over a period of 30 min. The basal tone (i.e. 1 g) returned during this washing procedure. Subsequently the muscarinic antagonist under test was added to the organ bath; 30 min later a second arecoline concentration-response curve was determined. At least three concentrations of each muscarinic antagonist were tested (a separate trachea was used for every concentration of antagonist). In control experiments it was demonstrated that if a second arecoline curve was constructed without antagonist incubation, that this curve was similar to the first arecoline curve (data not shown).

Effect of muscarinic antagonists on EFS (electric field stimulation)-induced smooth muscle contraction

The plastic holders, to which the tissues were attached, were equipped with platinum electrodes to facilitate EFS of tissues using a Grass S88 stimulator (Grass Medical Instruments, Quincy, MA, U.S.A.) connected to the electrodes. Prior experiments showed that an impulse voltage of 40 V, 1 ms, repeated every 100 s, was optimal, i.e. gave reproducible constant twitch contractions. Frequency-response curves were constructed using 5 to 160 Hz. From these frequency studies 20 Hz was chosen for subsequent studies. At this frequency it was possible to demonstrate increases and decreases of EFS-induced contraction.

Prior to the experiments, tracheae were equilibrated as mentioned above (1 h) and subsequently stimulated with EFS for 30 min (40 V, 20 Hz, 1 ms.). After this conditioning period the twitch contractions were stable. Thereafter cumulative concentration-response curves were determined for different muscarinic receptor antagonists. Each antagonist addition was followed by 5 stimulation periods. Cumulative concentration-response curves were finalised with the concentration of antagonist that inhibited the EFS-induced contraction totally.

Effect of muscarinic antagonist inhalation on lung function parameters

BALB/c mice were anaesthetized with pentobarbitone sodium (Abbott Laboratories, North Chicago, IL, U.S.A.) (50 mg kg^{-1} body weight, i.p.) An anaesthesia-induced fall in body temperature was avoided by placing the mice in a heated chamber that kept their body temperature at around 37°C. Pulmonary function parameters were measured in these anaesthetized and spontaneously breathing mice using a modification of previously published technique for lung function measurements in guinea-pigs (Folkerts & Nijkamp, 1985; Folkerts *et al.*, 1988). The animals were prepared for measurement of airway resistance (R_L) and dynamic lung compliance (C_{dyn}) as described elsewhere (Folkerts & Nijkamp, 1985; Folkerts *et al.*, 1988). Briefly, airflow ($\dot{V}$) and tidal volume (V_t) were determined by cannulating and connecting the trachea with a flow head to a Gould Godart Pneumotachograph (Bilthoven, The Netherlands). A Validyne MP45-2 (Validyne Corp., Northridge, CA, U.S.A.) pressure transducer measured the transpulmonary pressure (P_{TP}) by determining pressure differences between the tracheal cannula and a cannula filled with saline inserted in the oesophagus.

Lung compliance (C_{dyn}) is the amount of lung expansion (volume change) per unit of pleural pressure change when the pressure and volume changes are measured at moments of zero flow. During breathing an additional pleural pressure change is needed to drive air in or out of the lungs. The ratio between this pleural pressure change and airflow rate is termed airway resistances (R_L). To eliminate the influence of lung expansion on the calibre of airways, R_L was determined from the pressure (ΔP) and flow differences ($\Delta \dot{V}$) at points of equal lung expansion during inspiration and expiration respectively. R_L and C_{dyn} were determined breath by breath by a modified method of Amdur & Mead (1958) using a computerized respiratory analyzer (Folkerts & Nijkamp, 1985). Briefly: dividing ΔP_L and $\Delta \dot{V}$ at isovolume points yielded the R_L ($\Delta P_L : \Delta \dot{V} = R_L$), and dividing ΔV_t and ΔP_{TP} between points of zero flow yielded the C_{dyn} ($\Delta V_t : \Delta P_{TP} = C_{dyn}$). The lung function parameters of the spontaneously breathing mice were determined 30 min after preparation of the animals.

Effects of inhalation of muscarinic antagonists on respiratory functions were determined as follows: muscarinic antagonists or control solution were nebulized with an ultrasonic nebulizer. Each concentration of antagonist or control solution was given by inhalation for 20 breaths leading to a constant reproducible effect. The procedure started with an inhalation of the nebulized control solution (PBS) followed by the lowest concentration of the antagonist 15 min later. This procedure was continued up to the highest concentration of the antagonist tested. Between each different concentration of the antagonist tested, murine ventilation was stable for at least 15 min. Changes in airway resistance and lung compliance were calculated and expressed as Δ airway resistance or Δ lung compliance. Negative Δ values indicate a decrease and positive Δ values an increase of the parameter

as compared to the lung function measured after inhalation of the nebulized control solution.

Data analysis

Competitive antagonism in the arecoline-induced contraction experiments was assessed according to the method of Arunlakshana & Schild (1959). pA_2 values (affinity) were determined by 2 methods: (1) pA_2 values were calculated for each concentration of the antagonists used according to $pA_2 = -\log \{[\text{antagonist}]/(DR-1)\}$ and the mean value (± s.e.mean) was calculated; (2) pA_2 values were denoted as the X-intercept of the Schild plot (Mackay, 1978). Schild plots were determined with a computer programme (Lotus) in which $P<0.05$ was chosen as the limit for fitting the Schild plot. The slope and correlation of the Arunlakshana-Schild plot-line were also calculated. When the correlation (r) is reliable ($P<0.05$) and the slope of the Schild plot equals unity, the interaction between antagonist and receptor is competitive. In this case the pA_2 value could be obtained directly from the X-intercept.

Inhibition of EFS-induced contraction of murine tracheae was expressed as a percentage of tracheal contraction prior to the addition of the muscarinic antagonist (i.e. 100%). The EFS-induced contraction was expressed as the mean effect of 5 stimulation-periods (± s.e.mean). From these cumulative inhibition curves, IC_{50} values (concentration of antagonist necessary to inhibit the EFS-induced contraction by 50%) were automatically calculated by computer.

Changes in lung function parameters were analysed statistically by the unpaired Student's t test. A P value of $<0.05\%$ was taken as being statistically significant.

Source of materials

Arecoline was obtained from de Onderlinge Pharmaceutische Groothandel (OPG), Utrecht, The Netherlands. Atropine was obtained from Sigma Chemical Company, St. Louis, U.S.A. The muscarinic receptor antagonists pirenzepine (PZ), 4-diphenyl acetoxy N-methyl piperidine (4-DAMP), and 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyridol[2,3-b][1,4]benzodiazepin-6-one (AFDX-116)) were gifts from Dr H.N. Doods, Dr Karl Thomae, GmbH, Div. of Molecular Pharmacology, Postfach 1755, D7950, Biberach, Germany. Pilocarpine and gallamine were gifts from Dr W. Vleeming, University of Utrecht, P.O. Box 80.082, 3508 TB, Utrecht, The Netherlands. All reagents used to make up the Krebs solution were of Analar quality.

Results

Effects of muscarinic receptor antagonists on arecoline-induced tracheal contractions

All antagonists used in the arecoline-induced tracheal contraction experiments shifted the arecoline concentration-response curves to the right in a concentration-dependent fashion and over a large concentration-range (Figure 1). None of the antagonists used depressed the maximum contraction significantly, even at the highest concentrations (Table 1). Figure 2 presents Schild plots for the 4 different antagonists used and Table 2 gives affinity (pA_2) values, slope factors, and correlation values. Affinities were highest for atropine ($pA_2 = 8.61 \pm 0.24$; mean ± s.e. mean) and 4-DAMP ($pA_2 = 8.68 \pm 0.26$; mean ± s.e. mean). The muscarinic receptor antagonists suppressed the arecoline-induced tracheal contractions with the following rank order of potency: atropine = 4-DAMP > PZ = AFDX-116. pA_2 values of PZ and AFDX-116 were both significantly ($P<0.05$) lower as compared to the pA_2 values of atropine and 4-DAMP.

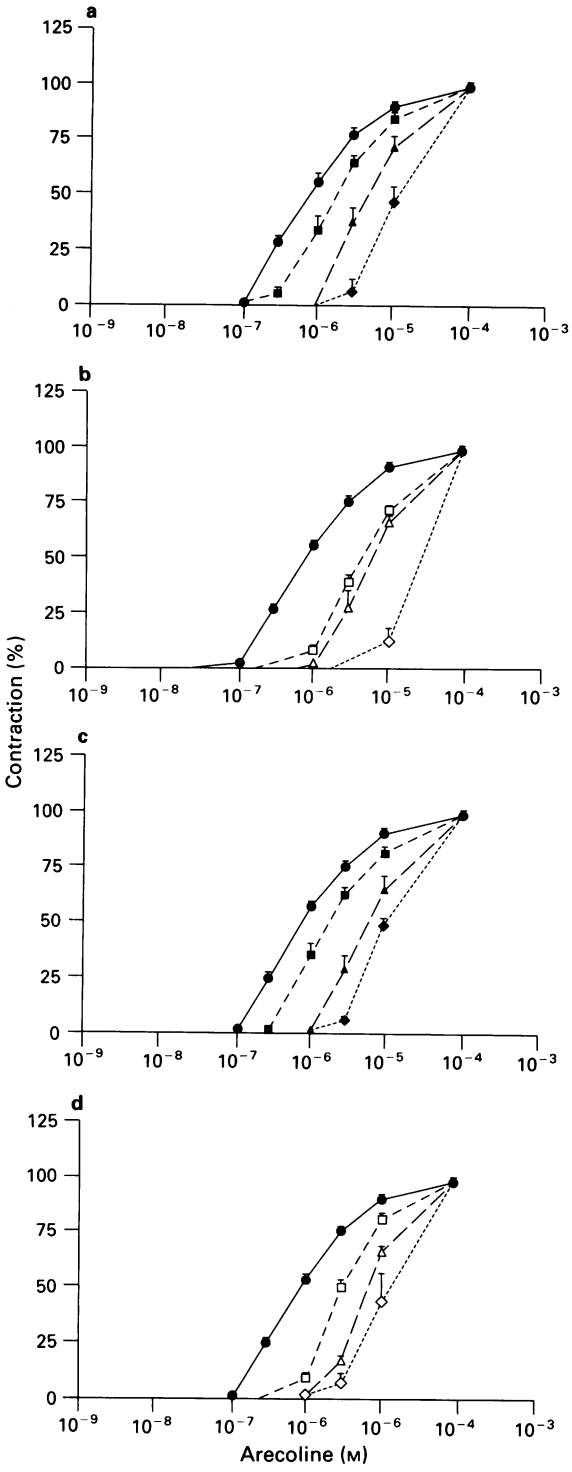

Figure 1 Effects of selective competitive muscarinic receptor antagonists on concentration-response curves for arecoline-induced contraction of murine isolated tracheae. An initial concentration-effect curve was performed for each experiment before the addition of each antagonist. The initial curves are shown as controls. Then, a second concentration-response curve was performed on the same tissues in the presence of antagonists, added 30 min earlier. Each concentration of antagonist (3 different concentrations) was tested on separate tracheae. Values are means (% of maximal contraction) + s.e. mean. $n = 5$ for each concentration of antagonist. (a) Effect of atropine; (b) effect of pirenzepine; (c) effect of 4-diphenyl acetoxy N-methyl piperidine (4-DAMP); (d) effect of AFDX-116. (●) Control; (■) 3×10^{-9} M; (▲) 10^{-8} M; (◆) 3×10^{-8} M; (□) 10^{-6} M; (△) 3×10^{-6} M; (◇) 10^{-5} M antagonist.

Table 1 Maximal contraction (E_{max}) of murine tracheae induced by 10^{-4} M arecoline in the absence or presence of maximal concentrations of antagonists

	E_{max} ± s.e. mean (mg)
Control	1602 (413)
+ Atropine (3×10^{-8} M)	1326 (372)
Control	1557 (326)
+ Pirenzepine (10^{-5} M)	1068 (234)
Control	1437 (190)
+ 4-DAMP (3×10^{-8} M)	1184 (182)
Control	1069 (169)
+ AFDX-116 (10^{-5} M)	933 (221)

$n = 5$ for each group.

Effects of muscarinic receptor antagonists on EFS-induced tracheal contractions

EFS-induced tracheal contraction was frequency-dependent (Figure 3). From these experiments 20 Hz was chosen as the frequency for subsequent experiments investigating the inhibition of EFS-induced contractions by muscarinic antagonists. The basal EFS-induced contraction, prior to antagonist addition, was characterized as the 100% effect (469.36 ± 63.40 mg). Figure 4 shows an example of a trace showing the inhibition of EFS-induced contractions by cumulative addition of PZ. All antagonists tested inhibited the EFS-induced contraction in a concentration-dependent manner (Figure 5). IC_{50} values for the antagonists used are given in Table 3. The muscarinic receptor antagonists used suppressed the EFS-induced tracheal contractions with the following rank order of potency: 4-DAMP = atropine > PZ > AFDX-116.

Effects of muscarinic receptor antagonists on lung function parameters

In the absence of any treatment with inhaled antagonist the values of airway resistance and compliance were 0.51 ± 0.12 $cmH_2O\ ml^{-1}\ s$ and 0.08 $ml\ cmH_2O^{-1}$ respectively. Inhalation of 4-DAMP induced a significant decrease in airway resistance and an increase in pulmonary compliance (Figure 6). In contrast to the results from *in vitro* experiments AFDX-116 induced bronchoconstriction, seen as a significant increase in airway resistance. At the concentrations tested, AFDX-116 did not however change pulmonary compliance (Figure 7).

PZ and pilocarpine did not significantly affect pulmonary function changes at the concentrations tested (10^{-10}–10^{-4} M). this was also true for atropine except for some minor decreases in airway resistance after inhalation of some lower concentrations (data not shown).

Discussion

Knowledge about cholinoceptors and especially muscarinic receptor subtypes in airway smooth muscle is available in many animal species such as guinea-pig, ox, dog, rat, rabbit and man, but not in mice (Fryer & Mclagan, 1984; Blaber *et al.*, 1985; Van Koppen *et al.*, 1985; Faulkner *et al.*, 1986; Eglen & Whiting, 1986; O'Rourke *et al.*, 1987; Madison *et al.*, 1987; Beck *et al.*, 1987; Bloom *et al.*, 1987; 1988; Roffel *et al.*, 1988; Ito & Yoshitomi, 1988; Minette & Barnes, 1988; Minette *et al.*, 1988; Maclagan & Faulkner, 1989). From results described previously it was concluded that carbachol and arecoline, both recognized as selective cholinoceptor agonists, can contract murine tracheal smooth muscle, indicating the presence of cholinoceptors on murine tracheae

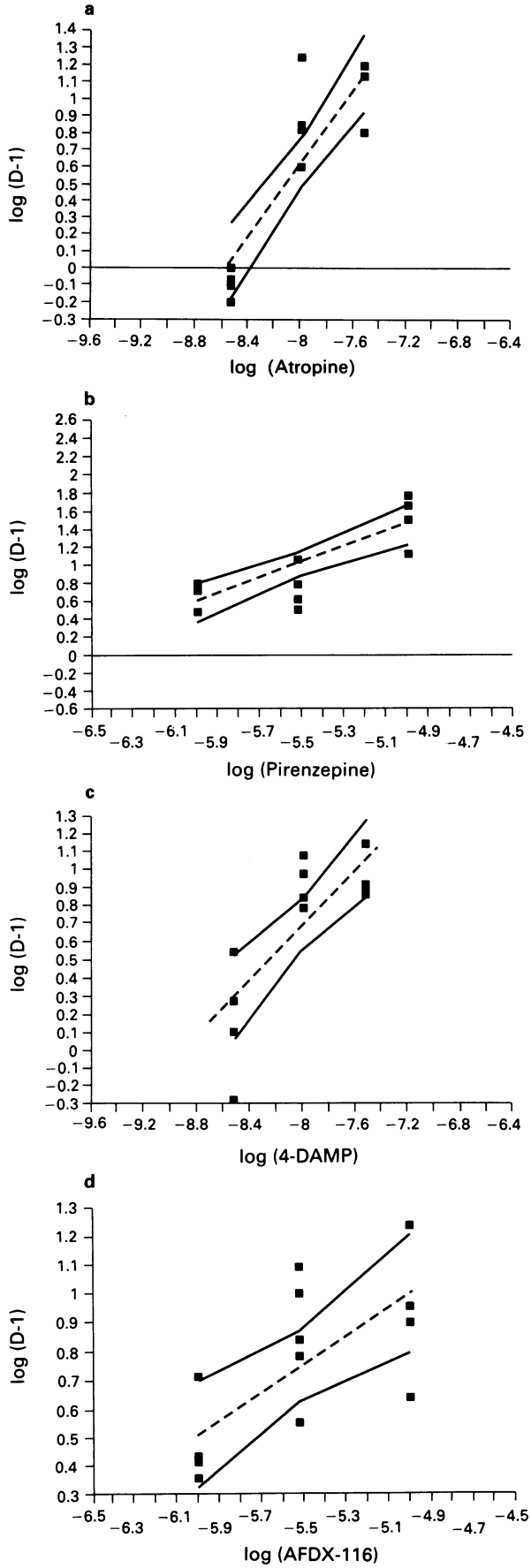

Figure 2 Arunlakshana-Schild plots for the antagonism of the arecoline-induced contraction of murine isolated tracheae by atropine (a), pirenzepine (b), 4-diphenyl acetoxy N-methyl piperidine, (4-DAMP) (c), and AFDX-116 (d). Individual data points (■) are presented (5 data points per concentration of antagonist). Regression lines were calculated with a reliability of $P < 0.05$. In the figures the Schild plot is demonstrated as the computerized line of best fit between upper ($P < 0.025$) and lower ($P < 0.025$) confidence limits.

Table 2 Inhibition of arecoline-induced contraction of murine tracheae (*in vitro*) by muscarinic receptor antagonists

Antagonist	*Mean* pA_2	pA_2 (Schild plot)	*Slope*	*Correlation* (r)
Atropine	8.61 (0.24)	8.56	1.09 (0.16)	0.88
Pirenzepine	6.52 (0.25)	6.68	0.86 (0.16)	0.83
4-DAMP	8.68 (0.26)	8.90	0.76 (0.16)	0.80
AFDX-116	6.28 (0.28)	7.04	0.49* (0.14)	0.71

Values are mean ± s.e. mean ($n = 15$ for each antagonist; $n = 5$ for each concentration). *Significantly different from unity; ($P < 0.05$).

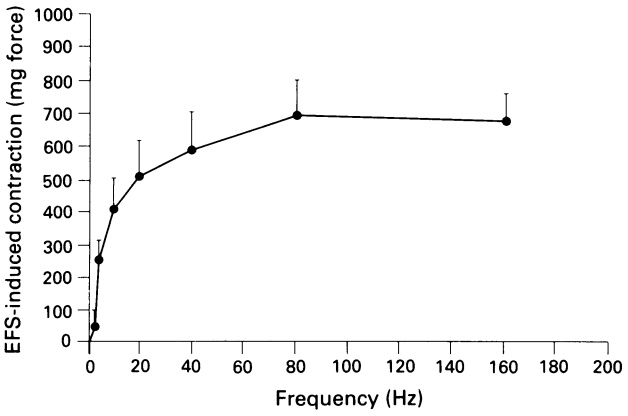

Figure 3 The effect of electric field stimulation (40 V, 1 ms, repeated every 100 s) at various frequencies from 0 to 160 Hz on murine isolated tracheae. Responses are shown as mean mg + s.e. mean ($n = 5$).

(Garssen *et al.*, 1990). In addition, we recently demonstrated the EFS can induce contraction of murine isolated tracheal smooth muscle (Van Oosterhout *et al.*, 1991).

In vitro studies in guinea-pig, ox and dog airway tissue showed that smooth muscle contraction to cholinoceptor agonists could be blocked strongly by 4-DAMP and less so by PZ. In addition, radioligand binding studies on bovine and human airway tissue confirm the low affinity of PZ for the muscarinic receptors present and the high affinity of 4-DAMP. Thus the muscarinic receptors on airway smooth muscle of most species investigated are predominantly M_3 receptors (Van Koppen *et al.*, 1985; Eglen & Whiting, 1986; O'Rourke *et al.*, 1987; Madison *et al.*, 1987; Roffel *et al.*, 1988). However, Roffel *et al.* (1988) demonstrated the existence of both M_2 and M_3 receptors in bovine tracheae using radioligand binding studies. By use of functional assays M_2 antagonists were shown to have a low affinity and M_3 antagonists a high affinity in this tissue. Bronchoconstriction in rabbits and dogs, induced by vagus nerve stimulation, can be blocked by PZ (Beck *et al.*, 1987; Bloom *et al.*, 1987; 1988). PZ has a low affinity for receptors on airway smooth muscle (Eglen & Whiting, 1986; O'Rourke *et al.*, 1987; Roffel *et al.*, 1988), thus excitatory M_1 receptors are most probably located on parasympathetic ganglia (Bloom *et al.*, 1988). M_2 muscarinic receptors are characterized as presynaptic autoreceptors. These prejunctional receptors inhibit the release of acetylcholine from cholinergic nerves. This has been found to be true for cats, dogs, guinea-pigs, and man (Fryer & Mclagan, 1984; Blaber *et al.*, 1985; Faulkner, 1986; Ito & Yoshitomi, 1988; Minette & Barnes, 1988; Minette *et al.*, 1988).

Because it is thought that different muscarinic receptor subtypes may play a role in airway diseases (Barnes, 1986; 1989) and because mice fulfil the requirements for an immunopharmacological model to study airway reactivity, in the present study the potencies of the different selective muscarinic antagonists were determined. All the muscarinic receptor antagonists suppressed the arecoline-induced contraction of murine tracheae concentration-dependently.

Although the maximal contraction induced by arecoline (10^{-4} M), was not significantly suppressed by the antagonists tested, there was a tendency for suppression of the maximal contraction at the highest concentration of the antagonist used. Such a tendency has also been found for rats and guinea-pigs (Gies *et al.*, 1989). The arecoline concentration-response curves were shifted to the right in a parallel and concentration-dependent fashion indicating the competitive nature of the antagonists used. Atropine and 4-DAMP exhibited the highest potencies. Thus muscarinic receptors on murine tracheal smooth muscle responsible for contraction may be characterized as M_3 receptors. This is in agreement

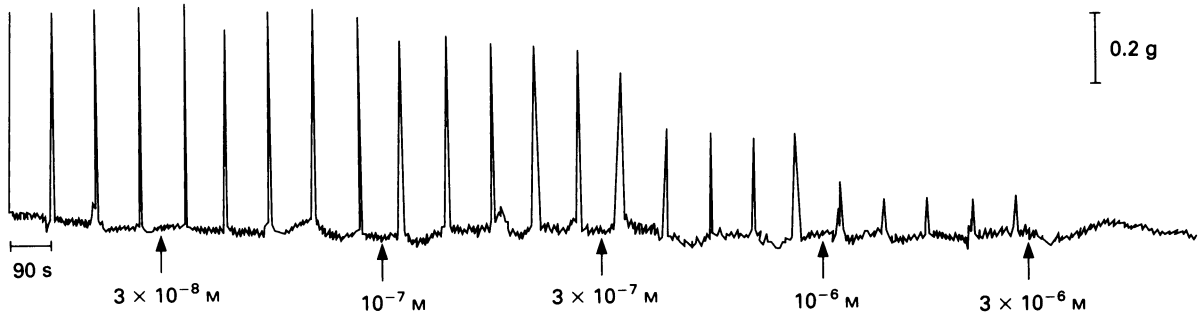

Figure 4 Representative tracing showing the effect of cumulative pirenzepine (PZ) addition on EFS (40 V, 20 Hz, 1 ms)-induced contraction of murine tracheae *in vitro*. Successive concentrations of PZ were added after each 5 stimulation periods (marked with an arrow).

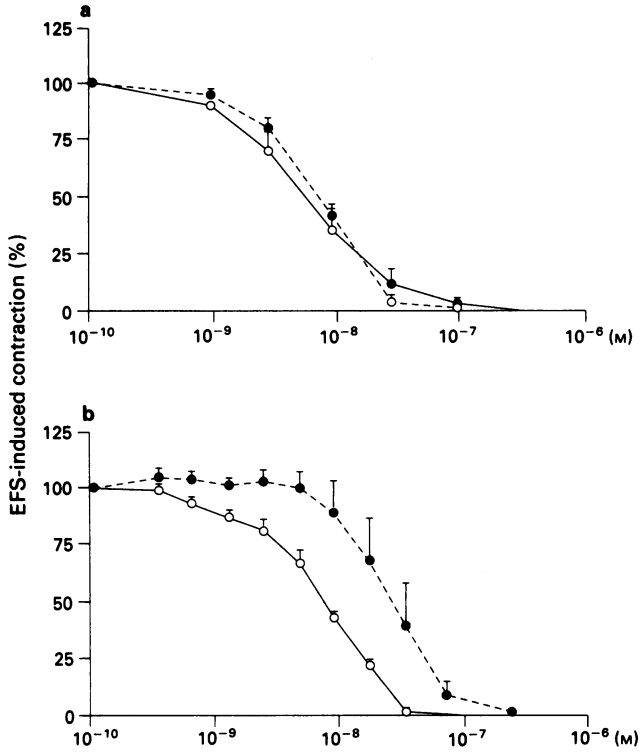

Figure 5 Inhibition of EFS (40 V, 20 Hz, 1 ms repeated every 110 s)-induced contraction of murine isolated tracheae by different muscarinic antagonists. Inhibition of EFS-induced contraction of murine tracheae is expressed as a percentage of tracheal contraction prior to the addition of the muscarinic antagonist (i.e. 100%). The EFS-induced contraction is expressed as the mean + s.e. mean of 5 successive stimulation-intervals (± s.e. mean. (a) Effect of atropine (●) and 4-diphenyl acetoxy N-methyl piperidine (○); (b) effect of pirenzepine (○) and AFDX-116 (●).

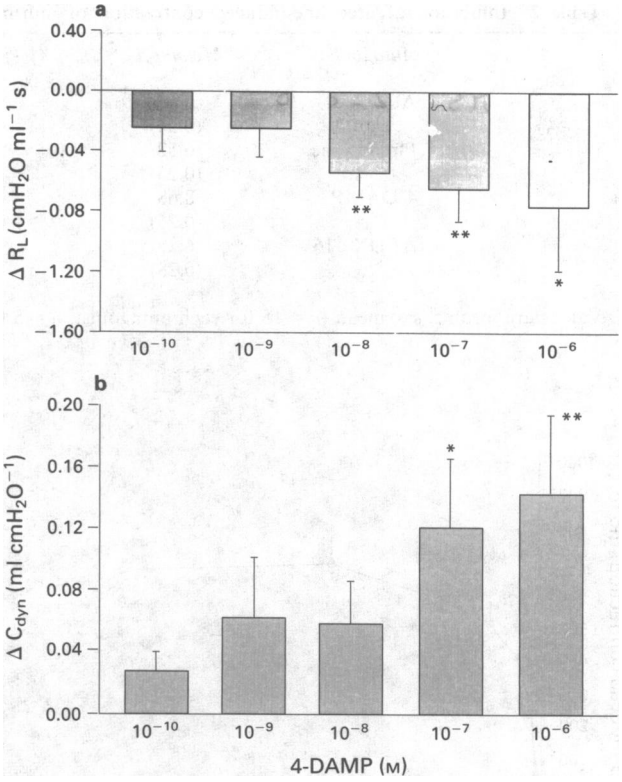

Figure 6 Effects of 4-diphenyl acetoxy N-methyl piperidine (4-DAMP) inhalation on respiratory function parameters in spontaneously breathing Balb/c mice. Each concentration of 4-DAMP or control solution was given by inhalation for 20 breaths. Changes in airway resistance and compliance were calculated and expressed as Δ airway resistance (R_L; a) or Δ lung compliance (C_{dyn}; b). Negative Δ values indicate a decrease and positive Δ values an increase of the parameter as compared to the lung function measured after inhalation of the nebulized control solution (PBS). Values are presented as the mean of 6 independent experiments + s.e. mean. $*P<0.05$; $**P<0.01$.

Table 3 Inhibition of EFS (40 V, 20 Hz, 1 ms, repeated every 100 s)-induced contraction of murine trachea by muscarinic antagonists (IC_{50} values)

Antagonist	IC_{50} (± s.e. mean)	$-\log IC_{50}$
Atropine	8.2×10^{-9} (1.2×10^{-9})	8.09
Pirenzepine	2.2×10^{-7} (3.1×10^{-8})	6.66
4-DAMP	7.5×10^{-9} (2.9×10^{-9})	8.13
AFDX-116	2.7×10^{-6} (1.0×10^{-6})	5.57

$n = 5$ for each antagonist.

with results in other animal species (Van Koppen *et al.*, 1985; O'Rourke *et al.*, 1987; Madison *et al.*, 1987; Roffel *et al.*, 1988). The potencies of AFDX-116 and pirenzepine were low as compared to both other antagonists. The pA_2 values that were found for murine tracheae agree very well with those reported for smooth muscle preparations from other animal species such as bovine and guinea-pig tracheal smooth muscle (Eglen & Whiting, 1986; Roffel *et al.*, 1988; Minette & Barnes, 1988). pA_2 values determined from the X-intercept of the Schild plots were similar except for a higher pA_2 value for AFDX-116. In this particular case the slope was also significantly different from unity ($P<0.05$). This could point to allosteric behaviour in murine tracheal smooth muscle or a lower selectivity of AFDX-116. In addition, the correlation-coefficient of 0.71 demonstrated that the X-intercept value for AFDX-116 was not very reliable in contrast to the good correlation values for the other three antagonists used, being higher than 0.80 (and thus significant at $P<0.05$). In summary, using arecoline-precontracted tracheae, we have demonstrated that the muscarinic receptor on murine smooth muscle cells can be characterized as predominantly M_3 receptors. For the study of receptors on other compartments such as nerves and/or ganglia, tracheae were precontracted by EFS. EFS induces release of endogeneous acetylcholine from nerve endings leading to smooth muscle contraction (Van Oosterhout *et al.*, 1991).

In prior studies we demonstrated that hexamethonium, which antagonizes ganglionic transmission, does not affect EFS-induced contractions. Thus in the EFS-model, ganglion stimulation does not play a role (Van Oosterhout *et al.*, 1991). Atropine and 4-DAMP inhibit EFS-induced contractions with a high efficacy. $-\log IC_{50}$ of AFDX-116 and PZ were low and the $-\log IC_{50}$ values for atropine and 4-DAMP were high. This is in agreement with data from the experiments with arecoline-induced tracheal smooth muscle contraction indicating the presence of M_3 receptors on murine trachea. In the EFS experiments the smooth muscle contraction is also indirectly M_3-dependent. Therefore M_3 receptors are probably located on smooth muscle cells.

M_2 autoreceptors could only be demonstrated in EFS-induced smooth muscle contraction experiments, because these receptors are located presynaptically on nerves. Pilocarpine, a selective agonist of M_2 receptors, inhibits cholinergic nerve-induced contraction of bronchi elicited by electric field stimulation, whereas it does not affect contraction induced directly by acetylcholine (Minette & Barnes, 1988). This

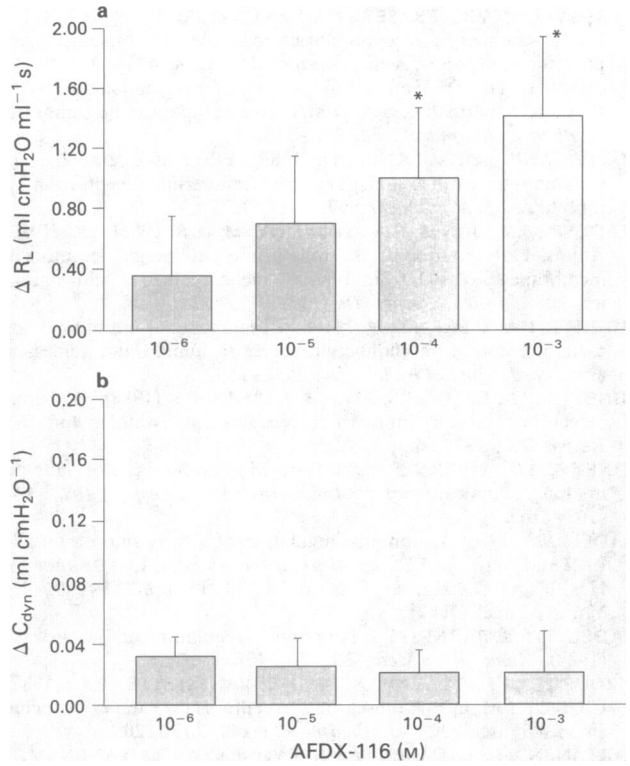

Figure 7 Effects of AFDX-116 inhalation on respiratory function parameters in spontaneously breathing Balb/c mice. Each concentration of AFDX-116 or control solution was given by inhalation for 20 breaths. Changes in airway resistance and compliance were calculated and expressed as Δ airway resistance (R_L; a) or Δ lung compliance (C_{dyn}; b). Negative Δ values indicate a decrease and positive 4 values an increase of the parameter as compared to the lung function measured after inhalation of the nebulized control solution (PBS). Values are presented as the mean of 6 independent experiments + s.e. mean. $*P<0.05$.

inhibitory effect can be blocked by gallamine (a non-competitive M_2 antagonist), confirming that the inhibition is mediated by M_2 receptors, which are presumably localized to postganglionic cholinergic nerves (Minette & Barnes, 1988). However in our experiments AFDX-116, a new selective M_2 receptor antagonist, did not induce inhibition of M_2 receptor function leading to an increase of EFS-induced contraction of murine tracheae. However, Figure 5 shows that small doses of AFDX-116 can have a tendency to increase EFS-induced contraction. In addition, AFDX-116 was less capable of inhibiting EFS-induced contractions than inhibiting arecoline-induced contractions, in which M_2 autoreceptors do not play a role. In rats there is also a controversy about the presence of M_2 receptors. Aas & Maclagan (1990) and Aas & Fonnum (1986) obtained evidence for muscarinic autoreceptors in pulmonary cholinergic nerves in albino rats. However, such receptors could not be detected in anaesthetized rats *in vivo* with gallamine as an antagonist (Fryer & Maclagan, 1984).

In contrast to the *in vitro* studies, AFDX-116 did change lung function measured *in vivo*, in that it increased pulmonary resistance significantly. This fits very well with the idea that M_2 receptors serve as autoreceptors responsible for the feed-back regulation of effects mediated by M_3 receptors that are directly responsible for smooth muscle contraction. Interestingly, inhalation of AFDX-116 had no effect on lung compliance. Because lung compliance depends in a major part on the elasticity of the alveolar walls, it can be suggested that M_2 receptors are located at sites higher in the airways such as the upper bronchi but not on the trachea, since in the *in vitro* trachea experiments it was not possible to increase EFS-induced tracheal smooth muscle contraction. Pilot experiments with gallamine demonstrated also that gallamine did not influence EFS-induced tracheal contraction. Inhalation of an M_2 agonist (pilocarpine) failed to decrease airway resistance. This may be due to the lower selectivity of this agonist as compared to the high selectivity of AFDX-116 for M_2 receptors. Pilocarpine has also some action at M_3 receptors. Interestingly 4-DAMP decreased airway resistance and increased lung compliance. This is in agreement with the theory that M_3 receptors, located on smooth muscle cells, are responsible for smooth muscle contraction. Inhibition of effects induced by M_3 receptor activation was detectable *in vivo* using the two lung function parameters, indicating the presence of M_3 receptors in lower and higher airways (upper bronchi/main bronchi) including the trachea as found in the *in vitro* trachea experiments. Effects of the other two antagonists tested, i.e. PZ and atropine, were almost nil except for some minor changes of airway resistance after atropine inhalation. These minor changes were characterized by a decrease in airway resistance at lower doses of atropine. The results with the two latter antagonists could be due to their lower selectivity. It could be argued that inhibition and stimulation of different receptor-mediated mechanisms negated their effects.

In summary it may be concluded that in murine airway smooth muscle, contraction can be induced by activation of muscarinic M_3 receptors. This phenomenon can be inhibited (feed-back regulation) by the activation of autoreceptors such as M_2 receptors. Using functional studies we have demonstrated the presence of M_3 receptors on murine tracheae and on other parts of higher (main bronchi) and lower murine airways. In contrast M_2 receptors could only be demonstrated in lung function studies *in vivo*. These studies demonstrated the presence of M_2 receptors at higher sites of murine airway such as the upper/main bronchi but taking the *in vitro* results into account, not on the trachea. Finally it may be concluded that a balance between M_3 and M_2 receptors plays an important role in the regulation of airway calibre in mice.

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