British Journal of Pharmacology (2008) 154, 1611–1618

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Isolated porcine bronchi provide a reliable model for development of bronchodilator anti-muscarinic agents for human use

G D'Agostino[1], AM Condino[1], L Gioglio[2], F Zonta[1], M Tonini[3] and A Barbieri[1]

[1]*Department of Experimental and Applied Pharmacology, University of Pavia, Italy;* [2]*Department of Experimental Medicine, University of Pavia, Italy and* [3]*Department of Physiological and Pharmacological Sciences, University of Pavia, Italy*

Background and purpose: In human airways, muscarinic acetylcholine receptors (mAChRs) exert a predominant role in the control of airways resistance and anti-muscarinic agents are currently included in the pharmacological treatment of chronic obstructive pulmonary disease (COPD). However, the development of more effective mAChR antagonists is hampered by considerable species variability in the ultrastrucural and functional control of airway smooth muscle, making extrapolation of any particular animal model questionable. This study was designed to characterize the mAChRs in a bronchial preparation from pigs, animals considered to provide close models of human biology.
Experimental approach: Smooth muscle bronchial strips were examined by electron microscopy in order to compare their neuromuscular structure with that of human bronchi and used to study the affinity of a series of selective mAChR antagonists, estimated as pK_is in competition binding assays with NMS and pA_2, by Schild analysis, in contractile experiments.
Key results: Pharmacodynamic binding parameters and affinity profiles of a series of antagonists were consistent with the presence of a majority of M_2 mAChRs along with a minor population of M_3 mAChRs. Functionally, the highly significant correlation between postjunctional pA_2 affinities and corresponding affinity constants at human recombinant M_1–M_5 subtypes indicated that smooth muscle contraction in porcine bronchi, as in human bronchi, was dependent on the M_3 subtype.
Conclusion and implications: Based on the characterization of mAChRs, isolated porcine bronchi provide an additional experimental model for development of mAChR antagonists for the treatment of human airway dysfunctions.
British Journal of Pharmacology (2008) **154,** 1611–1618; doi:10.1038/bjp.2008.208; published online 2 June 2008

Keywords: NMS binding; muscarinic antagonists; muscarinic receptor subtypes; pig airways; smooth muscle contraction

Abbreviations: AF-DX 250, dextrorotatory 11-(-1-piperdinyl]acetyl)-5,11-dihydro-6H-pyridobenzodiazepine-6-one; AQ-RA 741, 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one; 4-DAMP, 4-diphenylacetoxy-N-methyl-piperidine; DAU 5884, 8-methyl-8-azabicyclo-3-endooct-3-yl-1, 4-dihydro-2-oxo-3(2 H)-quinazoline carboxylic acid ester; HHSiD, hexahydrosiladifenidol; pF-HHSiD, *para*fluorohexahydrosiladifenidol; mAChR, muscarinic acetylcholine receptor; McN-A 343, 4-[[N-(3-chlorophenyl)carbamoyl]oxy]-2-butynyltrimethyl-ammonium chloride; MT3, mamba toxin-3; [^{3}H]NMS, [N-methyl-^{3}H]scopolamine methyl chloride; QNB, quinuclidinyl benzilate

Introduction

The parasympathetic (cholinergic) nervous system represents the main excitatory neural pathway in the airways of mammals as well as in humans (Racké and Matthiesen, 2004). It plays a predominant role in the control of distal airway resistance although the density of parasympathetic innervation is greatest in proximal airways and diminishes peripherally (Barnes, 1986). ACh released from cholinergic nerve terminals regulates airway functions (smooth muscle tone and mucus secretion) through stimulation of muscarinic ACh receptors (mAChRs; Alexander *et al.*, 2008). These receptors are members of the rhodopsin-like family of seven transmembrane receptors. Their predominant mode of signalling is mediated by the activation of GTP-binding proteins (G-proteins), although activation of other signalling molecules also occurs (Hall *et al.*, 1999). Five (M_1–M_5) different subtypes of mAChRs have been identified by molecular biological techniques, but so far a convincing pharmacological and functional characterisation has been provided for four of them (M_1–M_4) only. Nonetheless, there is now emerging evidence that the M_5 gene forms a functional M_5 mAChR (Eglen and Nahorsky, 2000).

Correspondence: Professor G D'Agostino, Department of Experimental and Applied Pharmacology, School of Pharmacy, University of Pavia, Viale Taramelli 14, I- 27100 Pavia, Italy.
E-mail: dagg@unipv.it
Received 16 January 2008; revised 7 April 2008; accepted 18 April 2008; published online 2 June 2008

The distribution of mAChRs in airways has been mapped by receptor autoradiography and *in situ* hybridization (Mak and Barnes, 1990; Mak *et al*., 1992; Hislop *et al*., 1998), and binding and functional studies revealed that at least three mAChR subtypes, namely M_1, M_2 and M_3, are expressed in airway smooth muscle and lung parenchyma of most mammals, including humans (Gies *et al*., 1989; Roffel *et al*., 1990; Patel *et al*., 1995; ten Berge *et al*., 1996). The presence of M_1 receptor mRNA was described in human (Bloom *et al*., 1988; Mak and Barnes, 1989; Mak *et al*. 1992) and pig peripheral lung (Haddad *et al*., 1994; Hislop *et al*., 1998), whereas M_2 and M_3 subtypes were found in smooth muscle preparations from these (Gies *et al*., 1989; Roffel *et al*., 1993; Haddad *et al*., 1994; Watson *et al*., 1995) and other species, including rodents and ruminants (see Eglen *et al*., 1996 for review). Under basal conditions, the M_3 receptor subtype is that involved in the bronchoconstriction action in all species. There is no evidence for expression of M_4 or M_5 receptors in human lung (Mak *et al*., 1992), although the presence of the M_4 receptor subtype is supported by *in situ* hybridization (Dörje *et al*., 1991a; Mak *et al*., 1993; Yasuda *et al*., 1993) and binding experiments in the rabbit (Lazareno *et al*., 1990) and pig lung (Chelala *et al*., 1998), indicating important species difference in the distribution and subtype of expressed receptors (Barnes, 2004).

Antimuscarinic agents are currently used in treating bronchoconstriction associated with chronic obstructive pulmonary disease and certain forms of asthma (Gosens *et al*., 2006), as an increase in cholinergic activity is a prominent pathophysiological mechanism in these conditions. Thus, mAChRs represent an attractive target for the development of novel antagonists that selectively block functional receptors in the effector cells; today, however, it is still a matter of controversy which animal species represents the best model for the search of bronchodilator antimuscarinic agents for human use (Barnes, 2004).

The present study was designed to assess whether the distribution and the function of mAChRs in the airways of the pig, an excellent animal model for biomedical research, closely resemble those observed in humans. For this purpose, strips of porcine bronchi, denuded of mucosa to minimize the influence of airway epithelium (Stuart-Smith and Vanhoutte, 1988; D'Agostino *et al*., 1990) were used. The neuromuscular structure of the preparation was assessed by electron microscopical techniques. A characterization of mAChRs subtypes was carried out with a series of mAChRs antagonists, investigated in radioligand binding and functional experiments. The comparison of the results obtained here with published morphological and pharmacological findings revealed a similarity of porcine bronchi to human bronchi. Based on this evidence, porcine bronchi appear an appropriate animal model for studying novel mAChR antagonists targeted to a more efficacious therapeutic control of airway diseases.

Methods

Tissue preparation

Lungs of mature large white pigs (>5 months, carcass weight 180–200 kg) were obtained from a local abattoir and rapidly transported to the laboratory. The bronchial tree was dissected and rings (5–6 mm I.D.) of segmental bronchi (third order) were isolated. The mucosa was removed from the tissue by rubbing the luminal surface with a moistened pipe cleaner. Histological inspection by light microscopy indicated that such procedure did not damage the basal membrane (not shown; see Klapproth *et al*., 1997). The muscularis was carefully lifted away from underlying cartilage and connective tissue by using a surgical knife. Strips (4 mm width, 15–18 mm length and 40–60 mg wet weight) were obtained from transverse rings of airway wall.

Electron photomicrography

Specimens of bronchial smooth muscle were fixed for 4 h at 4 °C in 3.5% glutaraldehyde, dissolved in Millonig's phosphate buffer at pH 7, washed in Millonig's buffer, postfixed in a 1% osmium tetroxide solution in Millonig's buffer for 1 h at 4 °C and dehydrated in ascending concentrations of ethyl alcohol. Specimens were then treated with propylene oxide, embebbed in Epon 812, and cut into semithin and ultrathin sections using a Reichert OM 12 ultramicrotone. Semithin sections for light microscopy were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined with a Zeiss 109 electron microscope.

Binding experiments

Bronchial strips, prepared as described above, were homogenized with an Ultraturrax at full speed for 30 s, followed by homogenization in a glass-on-teflon homogenizer in Na^+/Mg^{++} HEPES buffer pH 7.4. (100 mM NaCl, 10 mM MgCl2, 20 mM HEPES) (w/v final dilution 1:30). The homogenate was divided into 5 ml aliquots, and stored at −80 °C until use.

Displacement (competition) experiments were performed by incubating 980 μl of the homogenate (final tissue diluition 1:140) at 30 °C for 45 min in the presence of 0.3 nM [N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) and increasing concentrations of unlabeled compounds (mAChR antagonists) dissolved in the assay buffer (Na^+/Mg^{++} HEPES buffer pH 7.4). Incubation volume was 1 ml. The reaction was terminated by rapid filtration and washing (3 × 2 ml) of ice-cold buffer using an IH 110 Inotech cell harvester (type G-7 IH 201 glass filters, Inotech Biotechnologies, Basel, Switzerland). The filters were the placed in plastic vials containing 4 ml of Filter Count (PerkinElmer Life and Analytical Sciences, Milan, Italy) and counted for radioactivity by liquid scintillation analyzer (PerkinElmer Tri-Carb 2800 TR).

Specific binding of [^{3}H]NMS was defined as the binding displaceable by 1 μM QNB (3-quinuclidinylbenzilate, racemic mixture). Protein content was determined with Bio-Rad Protein Assay kit (Bio-Rad, Mississauga, ON, Canada) using bovine serum albumin as standard.

Contractile experiments

Porcine bronchi strips (4 mm wide and 15 mm length) were suspended isometrically and equilibrated for 60 min under a

resting tension of 10 mN in a 10 ml organ bath containing Krebs–Henseleit solution (composition in mM: NaCl 118, KCl 5.6, $CaCl_2 2H_2O$ 2.5, $MgSO_4 7H_2O$ 1.19, NaH_2PO_4 1.3, $NaHCO_3$ 25 and glucose 10), oxygenated (95% O_2 and 5% CO_2) and thermoregulated at 37 °C. Indomethacin (3 μM) was added to avoid the possible influence of prostanoids on contractile response (D'Agostino *et al*., 1990; Catalli *et al*., 2002). In each preparation, a cumulative concentration–response curve was determined twice, 60 min apart, for any mAChR receptor agonist: McN-A 343 (3–300 μM), carbachol (0.01–30 μM), bethanecol (0.1–300 μM), methacholine (0.01–100 μM), muscarine (0.01–30 μM), oxotremorine (0.01–30 μM) and muscarone (0.01–1 μM).

The antagonist properties of the mAChR blockers were evaluated on concentration–response curves produced by muscarone. After construction of the first muscarone concentration–response curve, a 30 min washout period was allowed to return the basal tone (that is, 10 mN). The preparation was then exposed for 30 min to one of the following antagonists: atropine (1–30 nM), pirenzepine (1–10 μM), AF-DX 250 (3–30 μM), methoctramine (1–10 μM), tripitramine (1–10 μM), HHSiD (0.03–1 μM), 4-DAMP (10–100 nM), DAU 5884 (10–100 nM), pF-HHSiD (0.1–10 μM) and AQ-RA 741 (0.3–3 μM). A second muscarone concentration–response curve was then carried out in the presence of the antagonist under test. Only one concentration of antagonist was tested on each preparation and at least three increasing concentrations were assayed in separate experiments. The pA_2 values were determined according to Arunlakshana and Schild (1959). Time-matched control experiments were carried out in the absence of antagonists.

Calculation and statistical analysis

The maximal binding site (B_{max}) and the equilibrium dissociation constant (K_D) of the ligand [^{3}H]NMS was determined by Scatchard analysis from the saturation curve. Competition binding data from ligand displacement curves were analysed using Prism programme (GraphPAD software version 5, San Diego, CA, USA) to provide IC_{50} s values and Hill coefficients (n_H). The best fit to one- or two-sites-binding model were determined as an *F*-test ($P<0.01$) on the residual variance. Significance testing of the Hill coefficient (n_H) was carried out with an unpaired Student's t-test. Values were considered significantly different when $P<0.05$. The affinity (K_i) values of competing compounds were calculated from IC_{50} values according to the Cheng–Prusoff equation ($K_i = IC_{50}\ [1+[D]K_D^{-1}$; Cheng and Prusoff, 1973) and converted to pK_i ($-\log K_i$).

In functional experiments, agonists' potency values were expressed as $-\log EC_{50}$, where EC_{50} indicates molar agonist concentration inducing 50% of the maximum effect of the curve evaluated by nonlinear curve fitting. Intrinsic activity (α) of an agonist was determined as percentage of muscarone maximal response. Antagonists affinity (pA_2) values were determined by the Schild regression analysis, using agonist concentration ratios determined at EC_{50} levels on concentration–response curves in absence and in presence of antagonist (Arunlakshana and Schild, 1959). Means ± s.e.m. and confidence limits at 95% probability were evaluated by using a computer program (PHARM/PCS, version 5).

Drugs and chemicals

Carbachol, bethanecol, methacholine, oxotremorine, 4-[[N-(3-chlorophenyl)carbamoyl]oxy]-2-butynyltrimethylammonium chloride (McN-A343), atropine, pirenzepine and indomethacin were purchased from Sigma Chemical Company (St Louis, MO, USA); 4-diphenylacetoxy-N-methyl-piperidine (4-DAMP), 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one (AQ-RA 741), muscarine, para-fluorohexahydrosiladifenidol (pF-HHSiD), HHSiD and methoctramine were purchased from Research Biochemicals, Natick, MA, USA); mamba toxin-3 (MT3) from Peptide International (San Diego CA, USA).

Quinuclidinyl benzilate (QNB) was synthesized at Dr Karl Thomae GmbH (Biberach, Germany); 8-methyl-8-azabicyclo-3-endooct-3-yl-1,4-dihydro-2-oxo-3(2H)-quinazoline carboxylic acid ester (DAU 5884) and dextrorotatory 11-(−1-piperdinyl] acetyl)-5,11-dihydro-6H-pyridobenzodiazepine-6-one (AF-DX 250) were provided by Boehringer Ingelheim Italy (Milan, Italy).

[N-methyl-^{3}H]-scopolamine (2.94 TBq $mmol^{-1}$ specific activity) was obtained from New England Nuclear (Boston, MA, USA). Tripitramine sesquifumarate and muscarone were a generous gift of Professor C Melchiorre (University of Bologna, Italy) and Professor C De Micheli (University of Milan, Italy), respectively. All drugs were dissolved in distilled water, with the following exceptions: indomethacin, AF-DX 250, HHSiD and pF-HHSiD were prepared in ethanol (stock solutions) and diluted further in distilled water.

Results

Electron microscopy study

The ultrastructural morphology of the bronchial smooth muscle strips, devoid of epithelium, was investigated by transmission electronic microscopy. The smooth muscle cells were arranged in bundles and separated by space containing an amorphous matrix. No ganglia were evident. Nerve fasciculi related to smooth muscle ran parallel to muscle bundles (Figure 1a) and close contacts with smooth muscle membrane were more reliably detected when muscle bundles were cut in cross-section (Figure 1b). Micrographic analysis also revealed that in porcine bronchi most nerve fibres presented axonal varicosities containing predominantly (along with few large granular vesicles) or exclusively small agranular vesicles. Their profile resembles the profile of cholinergic nerves in primates and humans (El-Bermani and Grant, 1975; Daniel *et al*., 1986).

Binding experiments

In crude membrane homogenates prepared from porcine bronchi in Na^+/Mg^{++} HEPES buffer, binding of 0.3 nM [^{3}H]NMS (888 KBq) was inhibited by 85 ± 5% by 1 μM QNB. Therefore, specific binding amounted to about 85% of total binding at a concentration of the radioligand near its K_D value (0.61 nM). The binding of [^{3}H]NMS was specific and

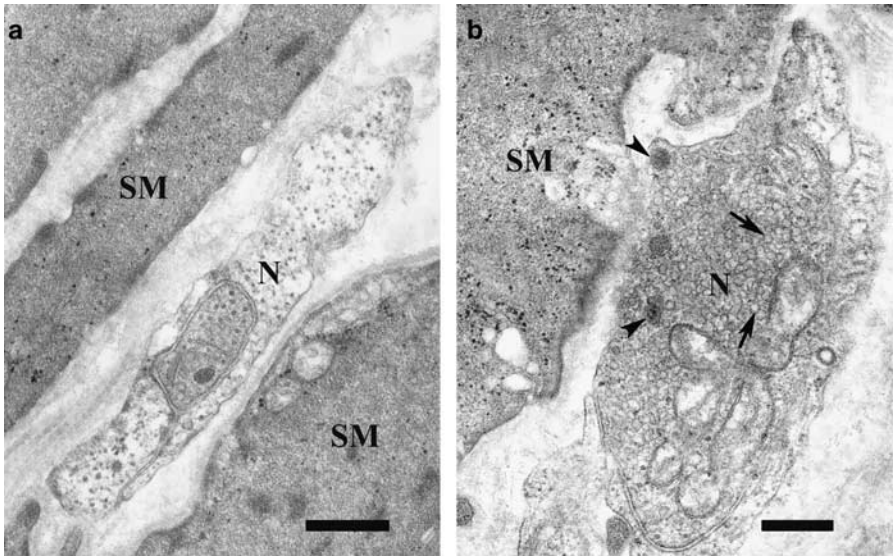

Figure 1 (**a**) TEM photomicrograph illustrating a nerve fibre (N) located between bronchial muscle cells (SM). Scale bar, 1 μm. Magnification, ×20000. (**b**) TEM photomicrograph showing nerve fibre (N) containing predominantly small agranular vesicles (arrows) and few large granular vesicles (arrowheads) in apposition to a smooth muscle cell (SM). Scale bar, 0.5 μm. Magnification, ×30000. TEM, transmission electronic microscopy.

Table 1 Binding parameters of mAChR ligands derived from displacement (competition) experiments against 0.3 nM [^{3}H]NMS in pig bronchi

Ligand	*$IC_{50}1$ (nM)*	*Expression (%)*	*$IC_{50}2$ (nM)*	*Expression (%)*	*pKi (high affinity)*	*pKi (low affinity)*	n_H	n
NMS	0.62 (0.57–0.65)	100			9.38		0.94 ± 0.07	6
Pirenzepine	551 (503–600)	100			6.43		0.89 ± 0.04	4
Tripitramine	1.83 (0.8–2.8)	78 (61–95)	119 (93–145)	22 (6–36)	8.91	7.10	0.78 ± 0.05*	6
Methoctramine	20 (9–31)	86 (80–92)	128 (98–158)	14 (8–20)	7.87	7.06	0.73 ± 0.06*	4
DAU5884	7.7 (1–14)	23 (14–32)	131 (89–173)	77 (65–89)	8.29	7.05	0.75 ± 0.03*	5
MT3	>1000				<6		ND	5

Abbreviations: DAU 5884, 8-methyl-8-azabicyclo-3-endooct-3-yl-1,4-dihydro-2-oxo-3(2 H)-quinazoline carboxylic acid ester; MT3, mamba toxin-3; NMS, [N-methyl-3 H]scopolamine methyl chloride; ND, not determined.
IC_{50} and expression values are the geometric means and 95% confidence limits (in brackets) of *n* (4–6) displacement experiments run in triplicate; n_H, Hill coefficient (n_H ± s.e.m.). *n_H significantly less than unity ($P<0.05$).

saturable (data not shown). Scatchard analysis of the saturation curve indicated that the non-selective antagonist bound to an apparently homogeneous population of mAChRs with a total density (B_{max}) of high-affinity binding sites of 425 ± 65 fmol (mg protein)$^{-1}$.

Pirenzepine (M_1 selective), DAU 5884 (M_1/M_3 selective), methoctramine (M_2/M_4 selective), tripitramine (M_2 selective) and MT3 (M_4 selective) were studied in competition experiments, testing the concentration-dependent inhibition of [^{3}H]NMS binding by increasing concentrations of the putative selective antagonists.

According to Scatchard and Hill plot analyses, the pirenzepine displacement-curve fitted a single-site model ($n_H = 0.89 \pm 0.04$; pKi = 6.43). At variance, nonlinear least squares regression analysis of occupancy–concentration curves of DAU 5884, methoctramine and tripitramine differed from a one-binding-site model. Hill coefficients significantly less than unit ($n_H = 0.75 \pm 0.03$, 0.73 ± 0.06 and 0.78 ± 0.05, respectively) could reflect the presence of a heterogeneous population of mAChRs.

DAU 5884 showed a best fit to a two-site model, with high affinity ($pK_i = 8.29$) and low affinity ($pK_i = 7.05$) for 23% and 77% of receptor sites, respectively. According to the two-binding-site model, methoctramine recognized with high affinity ($pK_i = 7.87$) 86% of binding sites and displayed low affinity ($pK_i = 7.06$) for the remaining 14%. A similar pattern was observed for tripitramine, the recognized proportion being equivalent to 78% ($pK_i = 8.91$) and 22% ($pK_i = 7.10$) of total bindings sites. As regards MT3, the peptide from green mamba venom, this peptide was completely inactive in the range of concentrations used (10–1000 nM).

The binding parameters of the mAChR antagonists used here are summarized in Table 1.

Functional experiments

A series of mAChR agonists induced concentration-dependent contraction of the smooth muscle bronchial strips. All the compounds behaved as full agonists, compared to muscarone, except oxotremorine and McN-A 343, which showed a maximal contractile response (E_{max}) of 80.04 ± 1.1 and 21.8 ± 6%, respectively. The rank order of potencies ($-\log EC_{50}$) was muscarone (6.9 ± 0.06; $n=6$) > oxotremorine (6.82 ± 0.09; $n=4$) > carbachol (6.38 ± 0.09; $n=5$) >

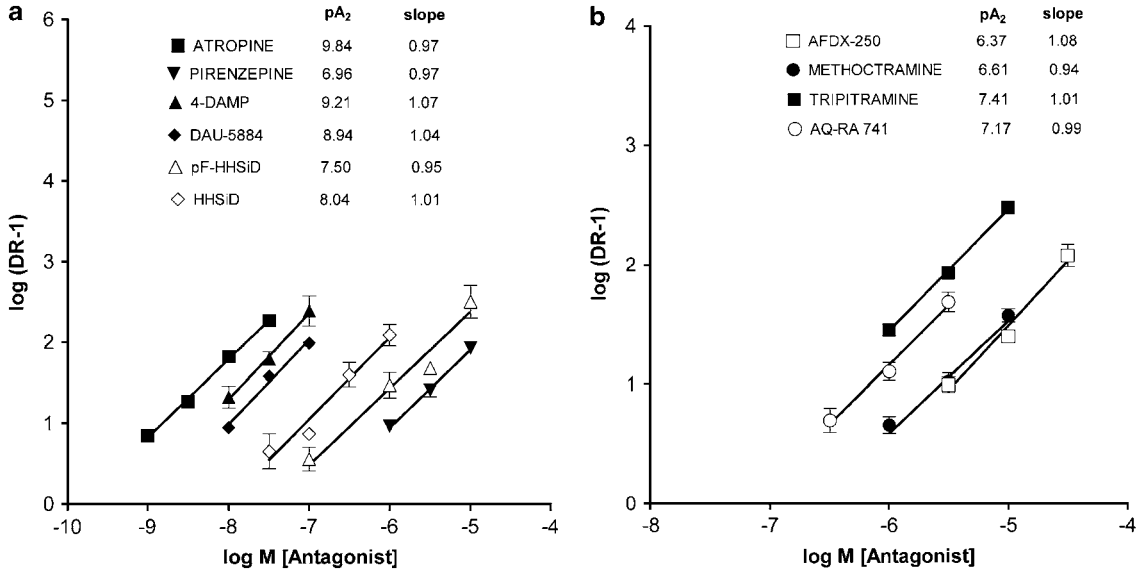

Figure 2 Schild plots for atropine and M_1/M_3 mAChR antagonists (**a**) and for M_2/M_4 mAChR antagonists (**b**). Each point represents the mean ± s.e.m. of 4–9 individual observations. The pA_2 values with slopes ± s.e.m. for the regression lines are also given in Table 2. mAChR, muscarinic acetylcholine receptor.

Table 2 Comparison of affinity (pK_i/pA_2) estimates of selective antagonists at human mAChR subtypes (pK_i) expressed in CHO cells and at porcine mAChRs (pA_2) in pig bronchi

Ligand	*CHO cells*					*Pig bronchi*	
	hM1	*hM2*	*hM3*	*hM4*	*hM5*	*post pA2*	*slope*
Atropine	9[c]	8.8[c]	9.82[d]	8.9[c]	9.2[c]	9.84 ± 0.05	0.97 ± 0.06
Pirenzepine	8.20[a]	6.65[a]	6.86[a]	7.43[a]	7.05[a]	6.96 ± 0.05	0.97 ± 0.01
Methoctramine	7.3[a]	7.88[a]	6.67[a]	7.50[a]	6.87[a]	6.61 ± 0.06	0.94 ± 0.16
Tripitramine	8.80[b]	9.57[b]	7.41[b]	8.19[b]	7.47[b]	7.41 ± 0.07	1.01 ± 0.06
AF-DX 250	6.37[a]	7.26[a]	6.16[a]	6.79[a]	5.52[a]	6.37 ± 0.04	1.08 ± 0.11
AQ-RA 741	7.54[a]	8.37[a]	7.2[a]	8.19[a]	6.08[a]	7.17 ± 0.03	0.99 ± 0.12
4-DAMP	9.24[a]	8.42[a]	9.28[a]	8.93[a]	8.98[a]	9.21 ± 0.07	1.07 ± 0.18
DAU 5884	8.95[g]	7.10[g]	8.86[g]	8.50[g]	8.15[g]	8.94 ± 0.04	1.04 ± 0.09
HHSiD	8.18[f]	6.60[d]	8.0[d]	7.68[e]	7.20[d]	8.04 ± 0.07	1.01 ± 0.14
pF-HHSiD	7.33[h]	6.56[h]	7.51[h]	7.24[h]	6.73[h]	7.50 ± 0.09	0.95 ± 0.13
MT3	6.7[c]	5.9[c]	6[c]	8.1[c]	6[c]	ND	

Abbreviations: AF-DX 250, dextrorotatory 11-(-1-piperdinyl]acetyl)-5,11-dihydro-6H-pyridobenzodiazepine-6-one; AQ-RA 741, 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one; CHO, Chinese hamster ovary; 4-DAMP, 4-diphenylacetoxy-N-methyl-piperidine; DAU 5884, 8-methyl-8-azabicyclo-3-endooct-3-yl-1,4-dihydro-2-oxo-3(2 H)-quinazoline carboxylic acid ester; HHSiD, hexahydrosiladifenidol; pF-HHSiD, *para*fluorohexahydrosiladifenidol; mAChR, muscarinic acetylcholine receptor; MT3, mamba toxin-3; ND, not determined.
Data from: (a) Dörje *et al.* (1991b); (b) Maggio *et al.* (1994); (c) Eglen and Nahorski (2000); (d) Buckley *et al.* (1989); (e) Chelala *et al.* (1998); (f) Guo *et al.* (1995); (g) unpublished data; (h) Hedge *et al.* (1997).

muscarine (6.33 ± 0.10; $n = 6$) > methacholine (6.07 ± 0.08; $n = 5$) > bethanecol (5.21 ± 0.05; $n = 6$) ≫ McN-A 343 (4.67 ± 0.6; $n = 4$).

The first and the second concentration–response curves of muscarone were superimposable. The muscarone concentration–response curve was shifted to the right in a parallel manner and without depression of the maximal response by atropine or subtype-preferring mAChR blockers, thus, indicating competitive antagonism as shown by Schild plot analysis (Figure 2). Postjunctional pA_2 values are listed in Table 2 and compared with affinity estimates for the same antagonists at human M_1–M_5 subtypes stably expressed in CHO (Chinese hamster ovary) cells. Their correlation analysis is shown in Figure 3. In Table 3, the postjunctional pA_2 values in pig bronchi are compared with corresponding pA_2/pK_B values obtained in human bronchi.

Discussion and conclusions

Acetylcholine exerts its effects on smooth muscle, nerves and secretory cells through activation of mAChRs (Barnes, 1987). These receptors were described on different target cells by means of different techniques, but the narrow 'selectivity window' of mAChR ligands used in the past generated some uncertainty about the expression of the mAChRs subtypes involved in the control of airway functions (see Racké and Matthiesen, 2004, for review). In this study, we used isolated bronchi of the pig, an excellent animal model for biomedical research, as a large body of evidence supports the notion that pigs and humans are similar in many aspects of both infant and adult anatomy, physiology, biochemistry, pathology and pharmacology (Hawarth and Hislop, 1981; Eglen *et al.*, 1996).

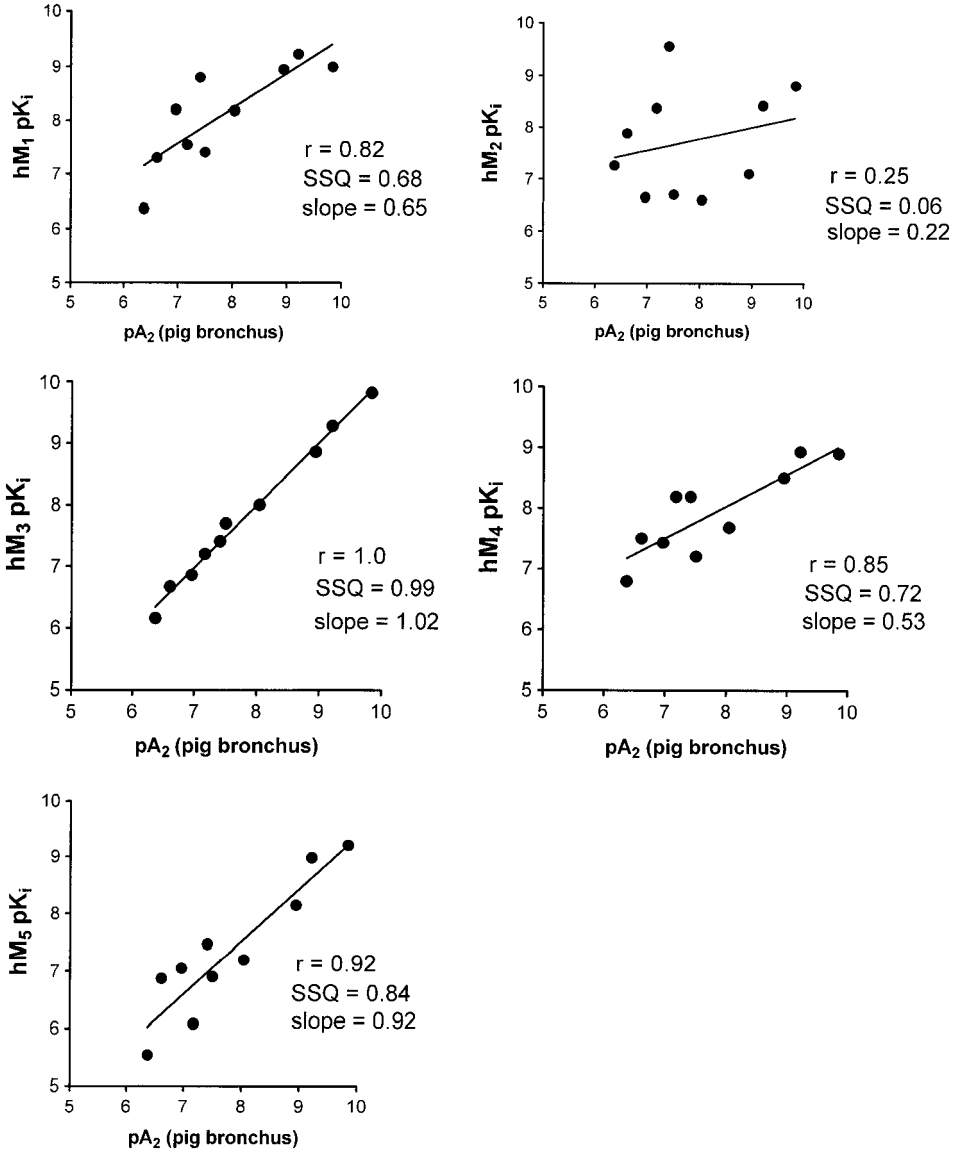

Figure 3 Correlation of postjunctional affinity (pA_2) values at mAChRs in pig bronchi with corresponding affinity constants (pK_i) at human cloned muscarinic receptor subtypes (hM_1-hM_5) expressed in CHO cells. r = correlation coefficient of Pearson; SSQ = sum of squares of differences between affinity estimates ($\Sigma(x-y)^2$). CHO, Chinese hamster ovary; mAChRs, muscarinic acetylcholine receptors.

In this respect, transmission electronic microscopy photomicrographs of sections of pig bronchial strips, offering a fine anatomical view of the neuromuscular structure, support the similarity of porcine with human bronchi (Daniel *et al.*, 1986; van Koppen *et al.*, 1988). The morphological analysis showed a preponderance of small agranular vesicles which are characteristic of cholinesterase-positive cholinergic nerve terminals, innervating the bronchial smooth muscle of large mammals and primates (El-Bermani and Grant, 1975; van Koppen *et al.*, 1987).

Based on this evidence, our strategy was to obtain an appropriate characterization of mAChRs in this bronchial preparation by means of a series of subtype-preferring mAChR antagonists. Their pharmacodynamic parameters, assessed in radioligand binding and functional experiments, are discussed in comparison with corresponding values reported in human recombinant mAChRs.

In binding assays, the non-selective ligand [^{3}H]NMS binds a homogeneous population of mAChRs yielding values in agreement with other non-selective mAChR antagonists tested in pig (Haddad *et al.*, 1994) and human airways (van Koppen *et al.*, 1985, 1988). With regard to the characterization of the mAChRs, the [^{3}H]NMS displacement curve for the M_1 antagonist pirenzepine was best fitted to a one-binding-site model with a pK_i of 6.43, a value close to that found for human cloned M_2 receptors (6.65; see Table 2, for comparison), thus excluding the participation of the M_1 receptor subtype. Conversely, a Hill coefficient significantly less than unity for DAU 5884 (M_1/M_3 selective), methoctramine (M_2/M_4 selective) and tripitramine (M_2 selective), suggests the presence of a heterogeneous expression of mAChRs in

Table 3 Comparison of postjunctional pA_2 estimates of mAChR antagonists in pig isolated bronchus with pA_2/pK_B values obtained in human bronchus

Ligand	*Pig bronchi* pA_2	*Human bronchi* pA_2/pK_B	*Selectivity profiles*
Atropine	9.84	9.1*	$M_3 > M_5 > M_1 \geqslant M_4 \geqslant M_2$
Pirenzepine	6.96	6.8*–6.8**	$M_1 > M_4 > M_5 \geqslant M_3 > M_2$
Methoctramine	6.61	5.3*–5.5**	$M_2 > M_4 > M_1 > M_3 = M_5$
Tripitramine	7.41	ND	$M_2 > M_1 > M_4 > M_3 = M_5$
AF-DX 250	6.37	ND	$M_2 > M_4 > M_1 > M_3 > M_5$
AQ-RA741	7.17	6.64**	$M_2 \geqslant M_4 > M_1 > M_3 > M_5$
4-DAMP	9.21	9.4*–8.96**	$M_3 \geqslant M_1 > M_4 = M_5 > M_2$
DAU 5884	8.94	8.57**	$M_1 \geqslant M_3 > M_4 > M_5 > M_2$
HHSiD	8.04	7.1*–7.09**	$M_3 > M_1 \geqslant M_4 > M_5 > M_2$
pF-HHSiD	7.50	6.7*	$M_3 \geqslant M_1 \geqslant M_4 > M_5 \geqslant M_2$

Abbreviations: AF-DX 250, dextrorotatory 11-(-1-piperdinyl]acetyl)-5, 11-dihydro-6H-pyridobenzodiazepine-6-one; AQ-RA 741, 11-[[4-[4-(diethylamino)-butyl]-1-piperidinyl]acetyl]-5, 11-dihydro-6H-pyrido(2, 3-b)(1,4)benzodiazepine-6-one; 4-DAMP, 4-diphenylacetoxy-N-methyl-piperidine; DAU 5884, 8-methyl-8-azabicyclo-3-endooct-3-yl-1,4-dihydro-2-oxo-3(2 H)-quinazoline carboxylic acid ester; HHSiD, hexahydrosiladifenidol; pF-HHSiD, *para*fluorohexahydro-siladifenidol; mAChR, muscarinic acetylcholine receptor; ND, not determined. Data from: *Watson *et al*. (1995); **Roffel *et al*. (1990).

porcine bronchi (Table 1). The high affinity values of methoctramine and tripitramine and the low affinity value of DAU 5884 are in agreement with the pharmacological profile of an M_2 receptor subtype, whereas the low affinity values of methoctramine and tripitramine and the high affinity value of DAU 5884 are consistent with the presence of a minor population of M_3/M_5 receptors (Table 2).

Based on these data, the dominant receptor subtype representing approximately 80% of receptor sites (Table 1), is an M_2 mAChR, a finding also corroborated by the high level of M_2 mRNA transcript (Haddad *et al*., 1994).

The presence of a putative M_4 mAChR subtype was excluded by the apparent inefficacy of MT3, used at concentrations that produce a complete blockade of the M_4 mAChR subtype. The MT3 peptide used in this study is known to be effective against the M_4 mAChR subtype as it showed activity in the human isolated detrusor (data not shown), a preparation which expresses these receptors (D'Agostino *et al*., 2000). In this respect, the absence of M_4 mRNA in porcine bronchi (Haddad *et al*., 1994) should be noted.

The identification of the minor M_3/M_5 receptor population was achieved with the use of previous (pirenzepine, methoctramine, tripitramine and DAU 5844) and additional mAChR antagonists (atropine, AF-DX 250, AQ-RA 741, 4-DAMP, HHSiD and pF-HHSiD) in functional contractile smooth muscle experiments. Porcine bronchi contracted following exposure to the mAChR agonists muscarone, carbachol, muscarine, methacholine and bethanecol, demonstrating full agonist properties, whereas oxotremorine and Mc-NA 343 behaved as partial agonists. The concentration–response curve induced by the most potent agonist, muscarone, was shifted in a competitive fashion by all the mAChR blockers with a slope not significantly different from unity, probably indicating the involvement of a single muscular mAChR subtype. The high affinity values obtained for atropine, 4-DAMP and DAU 5884, the intermediate affinity values for HHSiD, pF-HHSiD, pirenzepine and tripitramine, as well as the low affinity estimates for methoctramine and AF-DX 250 (see Table 2) suggest a prevalent M_3 mAChR pharmacological profile. Nevertheless, the participation of the M_5 receptor subtype could not be excluded *a priori*, given the similarity in M_3 and M_5 profiles (difference $<$ten-times) of the antagonists used. The use of AQ-RA741, a ligand with preferential affinity (difference $>$10-fold) for the M_3 over the M_5 receptor in animal and human tissues (Eglen and Nahorsky, 2000), strongly suggested that M_3 mAChRs mediate contractions of porcine bronchial smooth muscle. This conclusion is also supported by the finding of an excellent correlation between pA_2 values obtained in pig bronchi and those obtained in CHO cells expressing the human M_3 receptor (Figure 3c). The data generated in pig bronchi are also largely consistent with those obtained in human tracheal and bronchial preparations (Roffel *et al*., 1990; Watson *et al*., 1995) suggesting that the same mAChR subtype (that is, the M_3 subtype) is coupled to contraction. The difference in affinities observed with methoctramine, HHSiD and pF-HHSiD in human and pig bronchi could be explained in several ways. In human bronchi, methoctramine (3–300 μM) causes irregular rightward shifts of agonist concentration–effects curves accompanied by depression of the curve maximums, thereby, compromising estimation of its affinity. As far as HHSiD and pF-HHSiD are concerned, both compounds show some variability in pA_2 values for the M_3 receptor subtype, which are tissue and species-dependent. As such, the utility of these antagonists in muscarinic receptor classification is limited (Eglen *et al*., 1990).

In conclusion, isolated porcine bronchi seem to be a reliable model system to study mAChR function in peripheral airways and may represent an useful tool in the screening and development of novel bronchodilator agents for the therapy of airway diseases in humans.

Acknowledgements

This study was supported by funds from the University of Pavia (FAR-2006 and FAR-2007).

Conflict of interest

The authors state no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Arunlakshana O, Schild HO (1959). Some quantitative uses of drug antagonists. *Br J Pharmacol* **14**: 48–58.

Barnes PJ (1986). Neural control of human airways in health and disease. *Am Rev Respir Dis* **134**: 1289–1324.

Barnes PJ (1987). Cholinergic control of airway smooth muscle. *Am Rev Respir Dis* **136**: S42–S45.

Barnes PJ (2004). Distribution of receptor targets in the lung. *Proc Am Thorac Soc* **1**: 345–351.

Bloom JW, Halonen M, Yamamura HI (1988). Characterisation of muscarinic receptor subtypes in human peripheral lung. *J Pharmacol Exp Ther* **244**: 625–632.

Buckley NJ, Bonner TI, Buckley CM, Brann MR (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* **35**: 469–476.

Catalli A, Zhang D, Janssen LJ (2002). Receptors and signaling pathway underlying relaxations to isoprostanes in canine and porcine airway smooth muscle. *Am Physiol Lung Cell Mol Physiol* **283**: L1151–L1159.

Chelala JL, Kilani A, Miller MJ, Martin RJ, Ernsberger P (1998). Muscarinic receptor binding sites of the M4 subtype in porcine lung parenchyma. *Pharmacol Toxicol* **83**: 200–207.

Cheng YC, Prusoff WH (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108.

Daniel EE, Kannan M, Davis C, Posey-Daniel V (1986). Ultrastructural studies on the neuromuscular control of human tracheal and bronchial muscle. *Respir Physiol* **63**: 109–128.

D'Agostino G, Bolognesi ML, Lucchelli A, Vicini D, Balestra B, Spelta V *et al*. (2000). Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M_4 receptor subtype. *Br J Pharmacol* **129**: 493–500.

D'Agostino G, Chiari MC, Grana E, Subissi A, Kilbinger H (1990). Muscarinic inhibition of acetylcholine release from a novel *in vitro* preparation of the guinea-pig trachea. *Naunyn Schmiedebergs Arch Pharmacol* **342**: 141–145.

Dörje F, Levey AI, Brann MR (1991a). Immunological detection of muscarinic receptors subtypes proteins (m1-m5) in rabbit peripheral lung. *Mol Pharmacol* **40**: 459–462.

Dörje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR (1991b). Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J Pharmacol Exp Ther* **256**: 727–733.

Eglen RM, Hedge SS, Watson N (1996). Muscarinic receptor subtypes and smooth muscle function. *Pharmacol Rev* **48**: 531–565.

Eglen RM, Michel AD, Montgomery WW, Kunysz EA, Machado CA, Whiting RL (1990). The interaction of *para*-fluorohexahydrosiladifenidol at muscarinic receptors *in vitro*. *Br J Pharmacol* **99**: 637–642.

Eglen RM, Nahorsky SR (2000). The muscarinic M_5 receptor: a silent or emerging subtype? *Br J Pharmacol* **130**: 13–21.

El-Bermani AIW, Grant M (1975). Acetylcholinoesterase-positive nerves of Rhesus monkey bronchial tree. *Thorax* **30**: 162–170.

Gies JP, Bertrand C, Vanderheyden P, Waeldele F, Dumont P, Pauli G *et al*. (1989). Characterisation of muscarinic receptors in human, guinea pig and rat lung. *J Pharmacol Exp Ther* **250**: 309–315.

Gosens R, Zaagma J, Meurs H, Halayko AJ (2006). Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir Res* **7**: 73.

Guo ZD, Kameyama K, Rinken A, Haga T (1995). Ligand binding properties of muscarinic acetylcholine receptor subtypes (m1-m5) expressed in baculovirus-infected insect cells. *J Pharmacol Exp Ther* **274**: 378–384.

Haddad EB, Mak JC, Hislop A, Haworth SG, Barnes PJ (1994). Characterisation of muscarinic receptor subtypes in pig airways: radioligand binding and northern blotting studies. *Am J Physiol* **266**: L642–L648.

Hall RA, Premont RT, Lefkowitz RJ (1999). Heptahelical receptor signaling: beyond the G protein paradigm. *J Cell Biol* **145**: 927–932.

Hawarth SG, Hislop AA (1981). Adaptation of the pulmonary circulation to extra-uterine life in the pig and its relevance to the human infant. *Cardiovasc Res* **15**: 108–119.

Hedge SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM *et al*. (1997). Functional role of M_2 and M_3 muscarinic receptors in the urinary bladder of rats *in vitro* and *in vivo*. *Br J Pharmacol* **120**: 1409–1418.

Hislop AA, Mak JC, Reader JA, Barnes PJ, Haworth SG (1998). Muscarinic receptor subtypes in the porcine lung during postnatal development. *Eur J Pharmacol* **359**: 211–221.

Klapproth H, Reinheimer T, Metzen J, Münch M, Bittinger F, Kirkpatrick CJ *et al*. (1997). Non-neuronal acetylcholine, a signalling molecule synthezised by surface cells of rat and man. *Naunyn Schmiedeberg's Arch Pharmacol* **355**: 515–523.

Lazareno S, Bukley NJ, Roberts FF (1990). Characterisation of the muscarinic M_4 binding sites in in rabbit lung, chicken heart, and NG108 15 cells. *Mol Pharmacol* **38**: 805–815.

Maggio R, Barbieri P, Bolognesi ML, Minarini A, Tedeschi D, Melchiorre C (1994). Binding profile of the selective muscarinic receptor antagonist tripitramine. *Eur J Pharmacol* **268**: 459–462.

Mak JC, Barnes PJ (1989). Muscarinic receptor subtypes in human and guinea pig lung. *Eur J Pharmacol* **164**: 223–230.

Mak JC, Barnes PJ (1990). Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. *Am Rev Respir Dis* **141**: 1559–1568.

Mak JC, Baraniuk JN, Barnes PJ (1992). Localization of muscarinic receptor subtype mRNAs in human lung. *Am J Respir Cell Mol Biol* **7**: 344–348.

Mak JC, Haddad E-B, Bukley NJ, Barnes PJ (1993). Visualization of muscarinic m4 mRNAs and M_4-receptor subtypes in rabbit lung. *Life Sci* **53**: 1501–1508.

Patel HJ, Barnes PJ, Takahashi T, Tadjkarimi S, Yacoub MH, Belvisi MG (1995). Evidence for prejunctional muscarinic autoreceptors in human and guinea pig trachea. *Am J Respir Crit Care Med* **152**: 872–878.

Racké K, Matthiesen S (2004). The airway cholinergic system: physiology and pharmacology. *Pulm Pharmacol Ther* **17**: 81–198.

Roffel AF, Elzinga CR, Zaagsma J (1990). Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm Pharmacol* **3**: 47–51.

Roffel AF, Elzinga CR, Zaagsma J (1993). Cholinergic contraction of the guinea pig lung strip is mediated by muscarinic M_2-like receptors. *Eur J Pharmacol* **250**: 267–279.

Stuart-Smith K, Vanhoutte P (1988). Airways epithelium modulates the responsiveness of porcine bronchial smooth musle. *J Appl Physiol* **65**: 721–727.

ten Berge RE, Zaagsma J, Roffel AF (1996). Muscarinic inhibitory autoreceptors in different generations of human airways. *Am J Respir Crit Care Med* **154**: 43–49.

van Koppen CJ, Blankesteijn WM, Klassen AB, Rodriguez de Miranda JF, Beld AJ, van Ginneken CA (1987). Autoradiographic visualization of muscarinic receptors in pulmonary nerves and ganglia. *Neurosci Lett* **83**: 237–240.

van Koppen CJ, Blankesteijn WM, Klassen AB, Rodriguez de Miranda JF, Beld AJ, van Ginneken CA (1988). Autoradiographic visualization of muscarinic receptors in human bronchi. *J Pharmacol Exp Ther* **244**: 760–764.

van Koppen CJ, Rodriguez de Miranda JF, Beld AJ, Hermanussen MW, Lammers JW, van Ginneken CA (1985). Characterisation of the muscarinic receptor in human tracheal smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol* **331**: 247–252.

Watson N, Magnussen H, Rabe KF (1995). Pharmacological characterisation of the muscarinic receptor subtype mediating contraction of human peripheral airways. *J Pharmacol Exp Ther* **274**: 1293–1297.

Yasuda RP, Ciesla W, Wall SJ, Li M, Satkuss SA, Weisstein JS *et al*. (1993). Development of antisera selective for m4 and m5 muscarinic cholinergic receptors: distribution of m4 and m5 receptors in rat brain. *Mol Pharmacol* **43**: 149–157.