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M_1 and M_3 muscarinic receptors in human pulmonary arteries

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1 Acetylcholine (ACh) and the M_1 agonists (McN-A-343 or PD142505) relaxed human isolated pulmonary arteries which were pre-contracted with noradrenaline (10 μM). In preparations where the endothelium had been removed ACh induced a contractile response whereas the M_1 agonists (McN-A-343 or PD142505) had no effect.

2 ACh- and McN-A-343-induced relaxations were abolished after treatment of endothelium-intact preparations with the drug combination N^G-nitro-L-arginine (L-NOARG: 0.1 mM) and indomethacin (1.7 μM).

3 The affinity (pK_B value) for pirenzepine was higher in human pulmonary arteries when tissues were relaxed with McN-A-343 as compared with ACh (pK_B values, 7.71 ± 0.30 ($n=4$) and 6.68 ± 0.15 ($n=8$), respectively). In addition, the affinity for pFHHSiD against McN-A-343- and ACh-induced relaxations was 6.86 ± 0.13 ($n=3$) and 7.35 ± 0.11 ($n=9$), respectively.

4 The low affinities for methoctramine in human isolated pulmonary arteries with the endothelium either intact or removed, suggested the lack of involvement of M_2 and M_4 receptors in the ACh responses.

5 Phenoxybenzamine (3 μM: 30 min) abolished both ACh contraction and relaxation in human pulmonary artery. The ACh contraction was present when the phenoxybenzamine treatment was preceded by incubation with pFHHSiD (2 μM) but not with pirenzepine (1 μM). In addition, the ACh relaxation was present when preparations were treated with either pFHHSiD (2 μM) or pirenzepine (1 μM), before exposure to phenoxybenzamine.

6 These results in human isolated pulmonary arteries support the notion that only M_3 receptors, on smooth muscle, mediate the ACh-induced contraction whereas M_3 and M_1 receptors are involved in the endothelium-dependent ACh-induced relaxation.

Keywords: Human pulmonary arteries; muscarinic receptor; acetylcholine; McN-A-343; PD142505; pirenzepine; pFHHSiD; methoctramine; atropine; phenoxybenzamine

Introduction

The *in vivo* effects of acetylcholine (ACh) on the pulmonary vascular bed are dependent on the state of the vascular tone. Under normal vascular tone, ACh increased the lobar arterial pressure (Daly, 1957; Rose, 1957; Nandiwada *et al.*, 1983; El Kashef & Catravas, 1986; Matran *et al.*, 1991) whereas under increased vascular tone, ACh caused a decrease of this pressure (Fritts *et al.*, 1958; Nandiwada *et al.*, 1983; Hyman & Kadowitz, 1988). Since both responses are blocked by atropine, these effects appear to be induced by stimulation of muscarinic receptors. Similar observations have been made with a variety of isolated vascular preparations (Furchgott & Zawadzki, 1980; Altiere *et al.*, 1986). Furthermore, a pivotal role for the endothelium-derived relaxing factors, EDRF (Furchgott & Zawadzki, 1980) or nitric oxide (Gruetter *et al.*, 1979; Palmer *et al.*, 1987) and prostacyclin (Bunting *et al.*, 1976) has been shown to be involved in these responses.

In the lung there is a heterogeneity of muscarinic receptors and whether the opposite effects of ACh in the pulmonary vasculature are dependent on activation of distinct muscarinic receptor subtypes has not been resolved. In blood vessels the endothelium-dependent relaxations are mediated via activation of M_3 receptors in the rabbit aorta (Jaiswal *et al.*, 1991), the rabbit ear artery (Duckles & Garcia-Villalon, 1990) and the canine femoral artery (Rubanyi *et al.*, 1987). In contrast, different muscarinic receptors have been identified for the contractile response. In the bovine (Duckles & Garcia-Villalon, 1990) and pig coronary arteries (Van Charldorp *et al.*, 1988), ACh-induced contraction was mediated by activation of M_3 receptors. However, the latter study also described M_2 receptors involved in the pig cerebral arteries, data which were similar to those obtained in the rabbit aorta (Jaiswal *et al.*, 1991). M_1 receptors have also been associated with the vasoconstriction *in vivo* in mouse cerebral arterioles (Shimizu *et al.*, 1993) and in rabbit pulmonary vasculature (El Kashef & Catravas, 1991). Together these vascular results suggest that M_3 receptors located on the endothelium are associated with ACh-induced relaxation whereas the subtype of muscarinic receptor involved in ACh-induced contraction appears to be species and tissue-dependent.

While a number of studies have demonstrated both ACh-induced relaxation and contraction in human isolated pulmonary arteries (Joiner *et al.*, 1975; Greenberg *et al.*, 1987; Thom *et al.*, 1987; Crawley *et al.*, 1990), there have been no studies which identify the specific muscarinic receptor subtypes involved. Therefore, the aim of this study was to characterize pharmacologically the muscarinic receptors associated with the ACh-induced relaxation and contraction as well as to establish the contribution of products of the cyclo-oxygenase and NO-synthase pathway in the ACh-induced responses in human pulmonary arteries.

Methods

Isolated preparations

Human lung tissue was obtained from patients (33 male and 5 female) who had undergone lobectomy or pneumonectomy for

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removal of lung carcinoma. The mean age was 61 ± 3 years. Pulmonary arteries (2 to 4 mm internal diameter) were carefully dissected free of adjoining connective tissue and lung parenchyma. The preparations were placed in Tyrode solution and cut into rings of 3 to 5 mm in length. Experiments were performed on 232 arterial preparations derived from 38 lung samples (n). The endothelium, in some pulmonary arterial rings was mechanically removed by inserting both smooth-edged arms of a dissecting forceps into the lumen of the vessel and gently rolling the moistened preparation between the surface of a forefinger and the forceps for 10 s without undue stretching. Histological confirmation of the presence or the absence of the endothelium was made with the silver nitrate staining procedure. All tissues were used within 1 to 12 h postsurgery.

Arterial rings were set up in 10 ml organ baths containing Tyrode solution (concentration mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5; pH 7.4; aerated with 5% CO_2 in O_2 and maintained at 37°C. The preparations were placed under an initial load of 1.5 g which ensured a maximal response to the agonists used. Isometric force displacement transducers (Narco F-60) and physiographs (Linseis) were used to record the changes in force. Before the different experimental protocols, all tissues were allowed to equilibrate for 90 min and the bath fluid was exchanged every 10 min with fresh Tyrode solution.

Effects of enzyme inhibitors and muscarinic antagonists

Relaxation studies: endothelium-intact preparations were incubated for 30 min with Tyrode solution (control) or enzyme inhibitor (indomethacin, N^G-nitro-L-arginine (L-NOARG) or muscarinic antagonist (pirenzepine, methoctramine, pFHHSiD, atropine) and subsequently contracted with noradrenaline (10 μM). When the contraction reached a plateau, arterial preparations were stimulated with increasing concentrations of ACh, McN-A-343 or PD142505 in a cumulative fashion. At the end of the muscarinic agonist concentration-effect curves, the preparations were challenged with histamine (0.1 μM) to verify the presence of a functional endothelium. The maximal relaxation of these preparations with papaverine (0.1 mM) was obtained at the end of the experimental protocols. In the same protocol, after an incubation with Tyrode solution, ACh or McN-A-343 was added to preparations in which the endothelium had been removed.

Contraction studies: endothelium-denuded preparations were contracted with ACh (0.1 mM). When the response reached a plateau, the bath fluid was exchanged at 10 min intervals until the preparations returned passively to their initial resting tone. The rings were then incubated for 30 min with Tyrode solution (control), enzyme inhibitor or muscarinic antagonist and subsequently cumulative concentration-effect curves to ACh, McN-A-343 or PD142505 were produced. The same experiments were performed with ACh and McN-A-343 on endothelium-intact preparations. In both preparations, at the end of the M_1 agonist dose-response curves, 0.1 mM ACh was added.

Tyrode solution (control), antagonist or inhibitor incubations were performed on different preparations derived from the same lung sample. These paired experiments were repeated in a number of lung samples (n). A slight relaxation (0.2 g) in basal tone during the 30 min incubation period was observed. In addition, antagonist or inhibitor treatments did not affect noradrenaline-induced contraction.

Receptor protection studies

Another approach to characterize muscarinic ACh receptor sybtypes in tissues, involved the use of a selective and reversible muscarinic antagonist followed by a non-selective alkylating agent (phenoxybenzamine). The alkylated receptors are irreversibly inactivated, leaving one subtype of receptor intact after washout of the reversible muscarinic antagonist used (Eglen *et al.*, 1994).

Relaxation studies Endothelium-intact preparations were incubated for 30 min with Tyrode solution, pirenzepine (0.1 or 1 μM) or pFHHSiD (0.2 or 2 μM). Subsequently these tissues were incubated for 30 min with Tyrode solution or phenoxybenzamine (3 μM). After these incubations, the bath fluid was

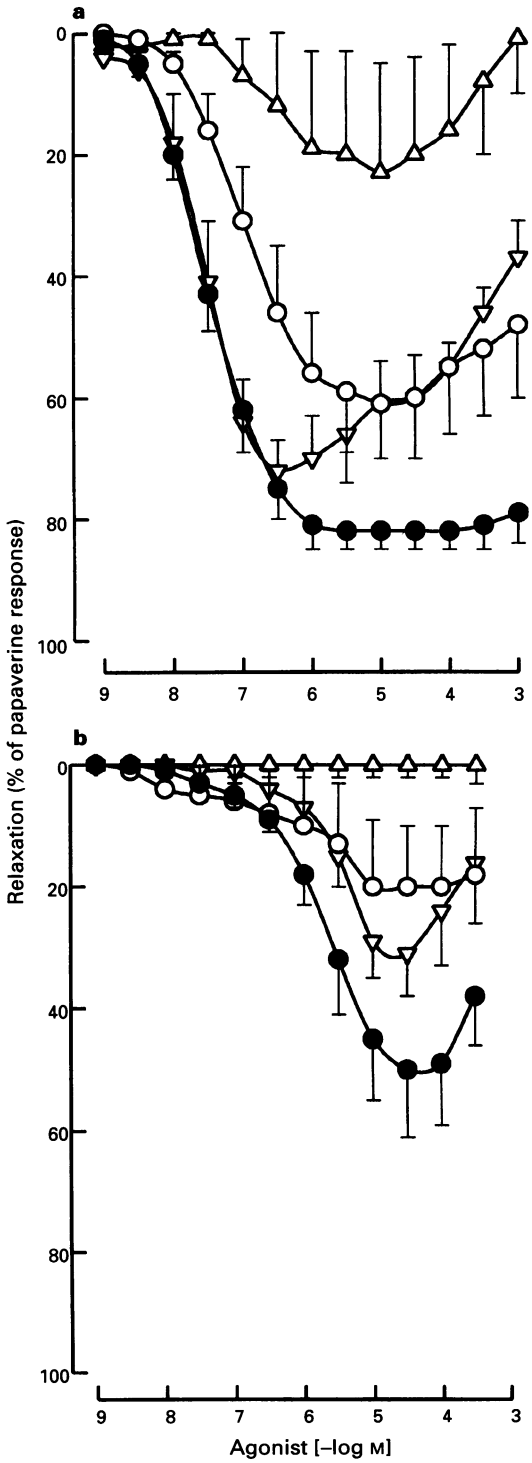

Figure 1 Relaxation of human endothelium-intact isolated pulmonary arteries induced by acetylcholine (a; $n=5$) or McN-A-343 (b; $n=4$) after 30 min incubation with either Tyrode solution (●), L-NOARG (0.1 mM; ▽), indomethacin (1.7 μM; ○) or a combination of both inhibitors at these concentrations (△). Each response is expressed as % of the papaverine (0.1 mM) relaxation. In (a) and (b), values are means ± s.e.mean (vertical lines) derived from paired lung samples, statistical analyses are presented in Table 2 and in Results section.

exchanged with fresh Tyrode solution (30 min) and a contraction was induced with the thromboxane A_2 mimetic U46619 (50 nM). When this response was obtained, arterial preparations were challenged with concentrations of ACh in a cumulative fashion. At the end of the ACh concentration-effect curves, the preparations were stimulated with papaverine (0.1 mM).

Contraction studies Preparations without an endothelium were incubated for 30 min with Tyrode solution, pirenzepine (0.1 or 1 μM) or pFHHSiD (0.2 or 2 μM). Subsequently these tissues were incubated for 30 min with Tyrode solution or phenoxybenzamine (3 μM). After these incubations, the bath fluid was exchanged with fresh Tyrode solution (30 min) and cumulative concentration-effect curves to ACh were produced. At the end of the ACh concentration-effect curves, the bath fluid was exchanged at 10 min intervals until the preparations returned passively to their initial resting tone. The preparations were then contracted with U46619 (50 nM).

Data analysis

The changes in force were measured from isometric recordings and expressed in grams (g). The relaxations produced with the cholinoceptor agonists were expressed as % of the relaxation induced with papaverine and the contractions as % of the maximal contractions induced with either ACh or U46619. The maximal response (E_{max}) produced with a cholinoceptor agonist and the EC_{50} values were interpolated from the individual concentration-effect curves. The EC_{50} values were transformed into pD_2 values, that is, the negative logarithms of EC_{50} values.

In order to determine the different muscarinic receptors present in the pulmonary arterial preparations, the affinities of the muscarinic antagonists (pK_B and pA_2 values) were calculated by use of displacement-curve experiments. When a significant difference for the pD_2 values in the presence of antagonist was observed, the equilibrium dissociation constant for the antagonist (K_B value) was calculated by use of the following equation: $K_B = [B]/(DR-1)$, where [B] is the con-

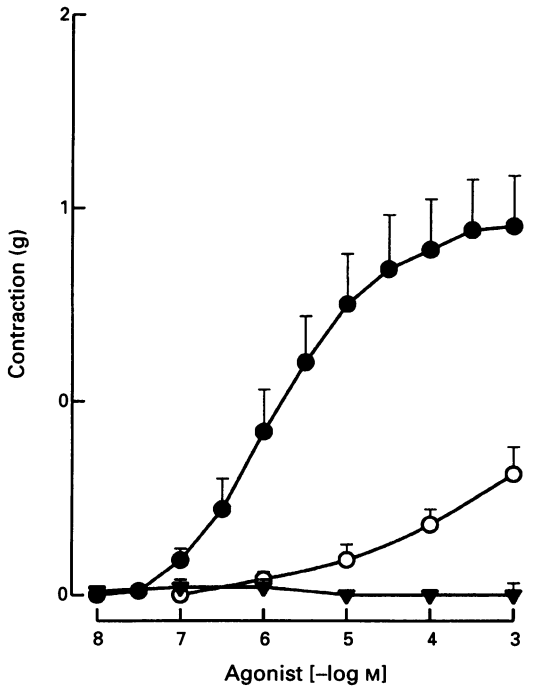

Figure 2 Acetylcholine contraction of human isolated pulmonary arteries in endothelium-removed preparations (●; $n=8$) or in endothelium-intact preparations (○; $n=5$) and effects of McN-A-343 in endothelium-removed preparations (▼; $n=6$). Values are means ± s.e.mean (vertical lines) expressed in grams (g).

Table 1 Effects of acetylcholine, McN-A-343 and PD142505 on human isolated pulmonary arteries with or without endothelium

	Relaxation experiments			*Contraction Experiments*		
Endothelium	(n)	E_{max} (%)	pD_2 values	(n)	E_{max} (g)	pD_2 values
			Acetylcholine			
Intact	(18)	67±05	7.31±0.09	(5)	0.44±0.12	4.65±0.28
Removed	(4)	03±03*	NC	(8)	1.00±0.13	5.80±0.21*
			McN-A-343			
Intact	(8)	49±05	5.65±0.05	(1)	0	NC
Removed	(1)	0	NC	(6)	0.03±0.02	NC
			PD142505			
Intact	(4)	34±06	5.44±0.24		NP	
Removed		NP		(4)	0.03±0.02	NC

The maximal responses (E_{max}) for the relaxations (endothelium-intact preparations) are expressed as % of the relaxation induced by papaverine (0.1mM) or as grams (g) for the contractions (endothelium-removed preparations. Values are means ± s.e.mean, (n) indicates the number of lung samples used, NC: not calculated and NP: not performed. *Indicates values significantly different from values obtained in endothelium-intact preparations.

Table 2 Effects of indomethacin and L-NOARG on the relaxations induced by acetylcholine or McN-A-343 in human pulmonary arteries with an intact endothelium

	Acetylcholine ($n=5$)		*McN-A-343* ($n=4$)	
	E_{max} (%)	pD_2 values	E_{max} (%)	pD_2 values
Control	87±03	7.42±0.12	51±11	5.66±0.08
Indomethacin	61±09*	6.90±0.21*	22±11	NC
L-NOARG	74±06*	7.61±0.15	31±07	NC
Indomethacin + L-NOARG	28±16*	NC	01±01*	NC

The endothelium-intact preparations were incubated for 30 min with Tyrode solution (control), or Tyrode solution containing indomethacin (1.7 μM) and/or L-NOARG (0.1 mM). The maximal responses (E_{max}) obtained with each agonist are expressed as % of the relaxation induced by papaverine (0.1 mM). Values are means ± s.e.mean, derived from paired lung samples (n). NC indicates not calculated and *indicates values significantly different from control.

centration of the antagonist and DR (dose ratio) is the ratio of EC_{50} of agonist in the presence and absence of antagonist. The K_B values were transformed into the pK_B values, that is, the negative logarithms of the K_B values. In the relaxation study with ACh, for each antagonist, 3–4 concentrations (2 concentrations at least per lung sample) were used to determine the pA_2 value according to the method of Arunlakshana & Schild (1959). For each lung sample, Schild plot analyses were performed, the slope and pA_2 value were determined by least square fitting of a regression line to the points.

All results are expressed as means ± s.e.mean. Statistical analysis was performed by Multirange ANOVA with a confidence level of 95%, while taking into account the preparations derived from the same or different lung samples (covariate).

Drugs

The drugs and their sources were: acetylcholine chloride, histamine dihydrochloride, indomethacin, L-NOARG (N^G-nitro-

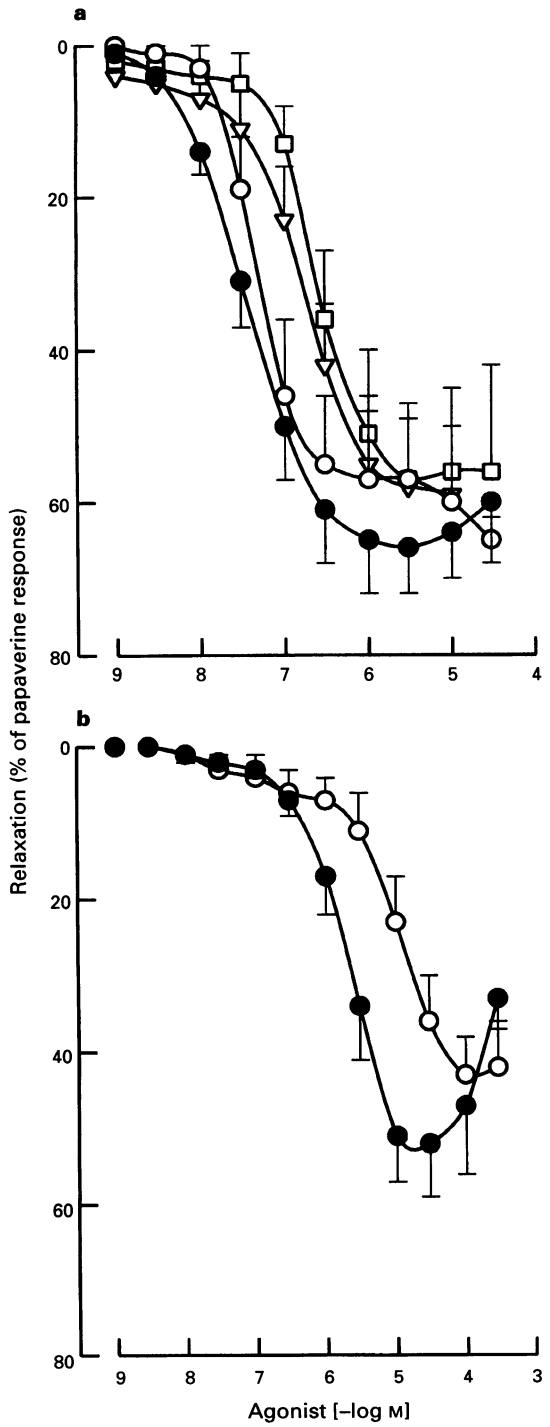

Figure 3 Relaxation of human endothelium-intact isolated pulmonary arteries induced by either acetylcholine (a; $n=4-10$) or McN-A-343 (b; $n=4$) after 30 min incubation with Tyrode solution (●) or pirenzepine (0.1 μM, ○; 0.5 μM, ▽ or 1 μM, □). Each response is expressed as % of the papaverine (0.1 mM) relaxation. Values are means ± s.e.mean (vertical lines); each curve is significantly different from appropriate control curve (ANOVA).

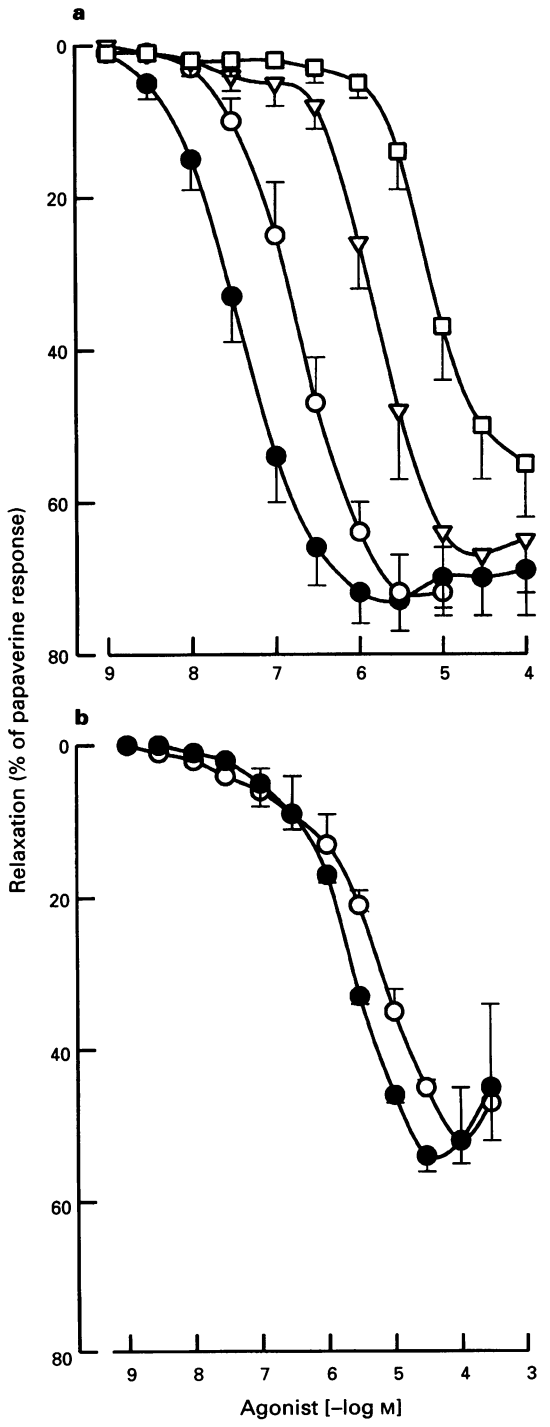

Figure 4 Relaxation of human endothelium-intact isolated pulmonary arteries induced by either acetylcholine (a; $n=3-9$) or McN-A-343 (b; $n=3$) after 30 min incubation with Tyrode solution (●) or pFHHSiD (0.2 μM, ○; 2 μM, ▽ or 8 μM, □). Each response is expressed as % of the papaverine (0.1 mM) relaxation. Values are means ± s.e.mean (vertical lines); each shifted curve is significantly different from appropriate control curve (ANOVA).

L-arginine), atropine sulphate, pirenzepine, U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$), noradrenaline (Sigma Chemical Co., St. Louis, MO, U.S.A.). pFHH SiD ((±)-p-fluoro-hexahydro-sila-difenidol hydrochloride), McN-A-343 ((4-hydroxy-2-butynyl)-1-trimethylammonium-*m*-chlorocarbanilate chloride), methoctramine, phenoxybenzamine (Research Biochemicals Inc., Natick, MA, U.S.A.). PD142505 (1-azabicyclo[2.2.1] heptan-3-one, O-[3-(3-methoxyphenyl)-2-propynyl]oxime, (Z)-(= +/)-, ethanedioate (1:1) (salt) (a gift of Dr Roy Schwartz, Parke Davis Pharmaceutical Research, Ann Arbor, MI, U.S.A.). Papaverine hydrochloride was obtained from Meram Laboratories (77020 Melun, France).

All agonists, antagonists and enzyme inhibitors were dissolved and diluted in Tyrode solution on the day the experiments were performed.

Results

Relaxation and contraction induced by cholinergic agonists

ACh, McN-A-343 and PD142505 produced concentration-dependent relaxations (Figure 1 and Table 1) of human endothelium-intact isolated pulmonary arterial rings contracted with noradrenaline (10 μM: 1.60±0.15 g; $n=26$). In some preparations, the high concentrations of cholinoceptor agonists induced small contractions (Figure 1). Removal of the endothelial layer eliminated the relaxations induced by either ACh or McN-A-343 (Table 1) and in these preparations ACh (0.1 μM and higher concentrations) induced a small and significant dose-dependent contraction ($E_{max}=0.26\pm0.15$ g, $n=4$) over the noradrenaline contraction. At the end of each experiment, the arterial ring preparations were relaxed with papaverine (0.1 mM: 1.73±0.14 g, $n=31$).

ACh induced concentration-dependent contractions in human pulmonary arteries with endothelium intact and removed are shown in Figure 2 and Table 1. In endothelium-removed preparations, no effects of McN-A-343 or PD142505 on basal tone were observed (Table 1); however, ACh (0.1 mM) always induced a contraction (0.36±0.06 g, $n=10$). In endothelium-intact preparations McN-A-343 was without any effect ($n=1$).

Effects of enzyme inhibitors

Indomethacin (1.7 μM) or L-NOARG (0.1 mM) significantly modified the ACh concentration-dependent relaxation curves (Figure 1a). However, the inhibition produced with indomethacin was effective on the threshold concentrations of ACh whereas L-NOARG inhibited the relaxation induced with the highest concentrations of ACh (>1 μM). The effects of each inhibitor on the E_{max} and the pD_2 values of the ACh-induced relaxation are shown in Table 2. In human pulmonary arteries treated with the combination of both inhibitors, the relaxation was abolished (Figure 1a and Table 2). Similar effects of these inhibitors against McN-A-343-induced relaxation were observed but only the combination of both inhibitors had a significant effect on the McN-A-343-induced relaxation curves (Figure 1b and Table 2).

The results obtained for ACh-induced contraction in endothelium-intact preparations treated with both indomethacin and L-NOARG were 6.00/6.18 (pD_2 values) with an E_{max}: 0.7/0.9 g ($n=2$). These values were similar to the ACh-induced contraction produced in preparations where the endothelium had been removed (Table 1).

Effects of muscarinic antagonists

Pirenzepine (0.5, 1, 10 or 100 μM) or pFHHSiD (0.2, 2 and 8 μM) significantly displaced the ACh concentration-dependent relaxation in a parallel manner (Figures 3a and 4a, respectively). The pA_2 values for these antagonists are presented in Table 3 and were derived from data presented in Figure 5. The lower concentration of pirenzepine (0.1 μM) significantly reduced the ACh-induced relaxation, this effect was observed at ACh concentrations between 0.5–10 μM (Figure 3a). Methoctramine (10, 50 or 100 μM) significantly shifted the relaxation curves to ACh, however, methoctramine (5 μM) was without any effect. Atropine (0.01, 0.03, 0.1 and 0.3 μM) also significantly shifted the ACh relaxation curves in a parallel

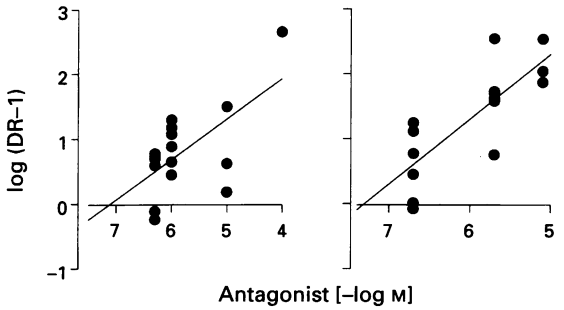

Figure 5 Schild-plots of log (dose-ratio (DR)−1) versus −log concentration of antagonist ((a) pirenzepine and (b) pFHHSiD). Each point is derived from the results shown in Figures 3a and 4a. However, additional data obtained with higher concentrations of pirenzepine are also shown. Analysis was based on pD_2 values calculated in treated preparations which were significantly different from control values. The linear regressions were performed by use of all data points and regressions were also performed through data derived from each lung sample. The calculated averages of pA_2 values and slopes are shown in Table 3.

Table 3 The pA_2 and pK_B values of muscarinic receptor antagonists on relaxation induced by acetylcholine or McN-A-343 in human pulmonary arteries with an intact endothelium

	Acetylcholine			*McN-A-343*
	pK_B	pA_2	*Slope*	pK_B
Pirenzepine	6.68±0.15 (8)	6.84±0.57 (7)	0.82±0.35	7.71±0.30 (4)
Methoctramine	5.37±0.24 (5)	5.23±0.20 (3)	1.86±0.79	NP
pFHHSiD	7.35±0.11 (9)	7.38±0.49 (7)	1.31±0.20	6.86±0.13 (3)
Atropine	9.37±0.03 (3)	9.14±0.36 (3)	1.27±0.20	NP

The acetylcholine relaxations were performed after an incubation period of 30 min with pirenzepine (0.5, 1, 10 or 100 μM), methoctramine (10, 50 or 100 μM), pFHHSiD (0.2, 2 or 8 μM) or atropine (0.01, 0.03, 0.1 or 0.3 μM). The McN-A-343-induced relaxations were produced after incubation with pirenzepine (0.1 μM) or pFHHSiD (0.2 μM). Schild plot slopes are not significantly different from unity. Values are means±s.e.mean, the numbers of lung samples used are shown in parentheses. NP indicates experiment not performed.

manner. Pirenzepine (0.1 μM) and pFHHSiD (0.2 μM) caused a parallel rightward shift of the McN-A-343 relaxation curves (Figures 3b and 4b). The pK_B value obtained with the antagonists against the ACh- or McN-A-343-induced relaxations are presented in Table 3.

Treatment of endothelium-removed preparations with pirenzepine (1 μM) or pFHHSiD (2 μM) caused a significant and parallel rightward shift of the ACh contraction curves whereas 10 fold lower concentrations of these antagonists did not affect these curves (Figure 6). Similar results were observed with

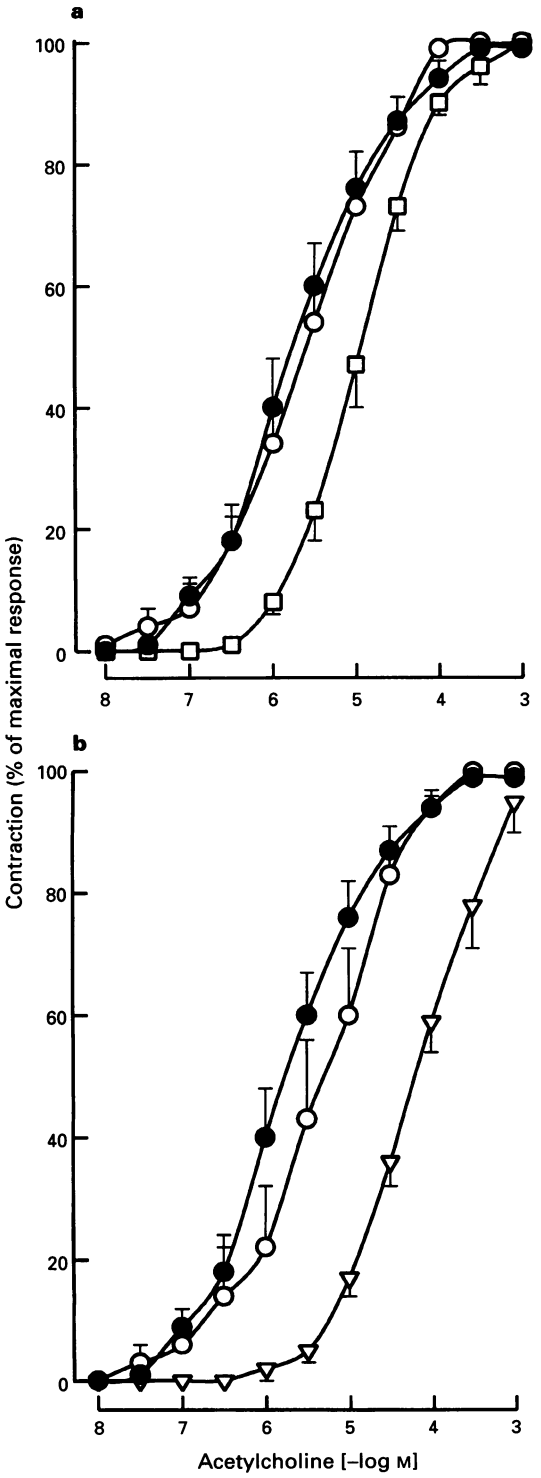

Figure 6 Acetylcholine contraction in human endothelium-removed isolated pulmonary arteries after 30 min incubation with either Tyrode solution (●), pirenzepine (a; 0.1 μM; ○ or 1 μM; □) or pFHHSiD (b; 0.2 μM; ○ or 2 μM; ▽). Each response is expressed as % of the maximal response induced by acetylcholine. In (a) and (b), values are means ± s.e.mean (vertical lines) derived from 5 paired lung samples. Each shifted curve (□ and ▽) is significantly different from the appropriate control curve (ANOVA).

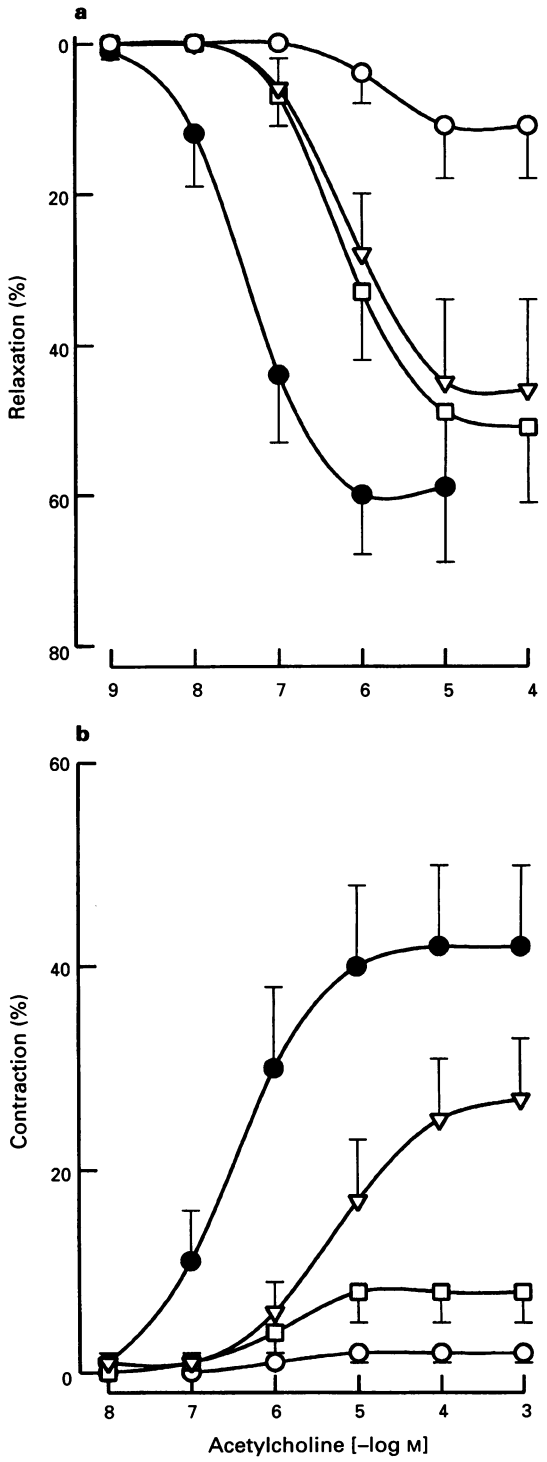

Figure 7 Effect of phenoxybenzamine (3 μM, 30 min) on acetylcholine responses in human endothelium-intact (a; $n=5$) and endothelium-removed (b; $n=4$) isolated pulmonary arteries after incubation with either Tyrode solution (○), pirenzepine (1 μM; □) or pFHHSiD (2 μM; ▽). Acetylcholine response after 1 h incubation with Tyrode solution (●). In (a) responses are expressed as % of the papaverine (0.1 mM) relaxation. In (b) responses are expressed as % of the U46619 (50 nM) contraction. Values are means ± s.e.mean (vertical lines) derived from paired lung samples. Statistical analysis are presented in Table 4.

Table 4 Effect of pirenzepine or pFHHSiD on the responses to ACh in phenoxybenzamine-treated human pulmonary arteries

Treatment	*Relaxation experiments* E_{max} (%) ($n=5$)	*Contraction experiments* E_{max} (%) ($n=4$)
Tyrode + Tyrode	62±09*	42±08
Tyrode + phenoxybenzamine	12±07	02±01
Pirenzepine 0.1 μM + phenoxybenzamine	37±07	08±03
Pirenzepine 1 μM + phenoxybenzamine	51±10*	08±03
pFHHSiD 0.2 μM + phenoxybenzamine	19±10	10±03
pFHHSiD 2 μM + phenoxybenzamine	47±11*	27±06§

The preparations, derived from (n) lung samples, were incubated for 30 min with Tyrode solution or a muscarinic antagonist and subsequently incubated (30 min) with Tyrode solution or phenoxybenzamine (3 μM). After these incubations, the bath fluid was exchanged with fresh Tyrode solution before the relaxation- or contraction-protocols. The ACh-induced relaxations were performed on endothelium-intact preparations pre-contracted with U46619 (50 nM). The maximal responses (E_{max}) were expressed as % of the relaxation induced by papaverine (0.1 mM). The ACh-induced contraction was performed on endothelium-removed preparations, E_{max} are expressed as % of the contraction induced by U46619 (50 nM). All values are means±s.e.mean. Statistical analysis: *$P<0.05$ versus (Tyrode + phenoxybenzamine) and §$P<0.05$ versus other treatments.

methoctramine 10 μM and 1 μM, respectively. The pK_B values obtained with these antagonists during the ACh-induced contraction were: pirenzepine, 6.75±0.31 ($n=5$); methoctramine, 5.69±0.15 ($n=5$) and pFHHSiD, 7.23±0.24 ($n=5$).

Receptor protection studies

ACh-induced relaxation or contraction responses were completely abolished by treatment of the vascular preparations with phenoxybenzamine (30 μM: 30 min). In endothelium-intact preparations, the relaxations induced by ACh were significantly protected when the preparations were pretreated with pirenzepine (1 μM) or pFHHSiD (2 μM) before addition of phenoxybenzamine (Figure 7a and Table 4). The ACh-induced contraction of endothelium-removed preparations was significantly protected against the phenoxybenzamine-effect only by pretreatment with pFHHSiD (2 μM; Figure 7b and Table 4).

Discussion

In human pulmonary arteries (present study), the data obtained with cholinoceptor agonists and the results from the phenoxybenzamine experiments suggest that ACh-induced relaxation is mediated by M_1 and M_3 receptors. Both of these muscarinic receptors are present on human pulmonary endothelial cells while only M_3 receptors were detected on the vascular smooth muscle. This latter observation is supported by data obtained with the selective M_1 agonists, McN-A-343 (Armstead *et al.*, 1988; Hu & El-Fakahany, 1990) and PD142505 (Schwarz *et al.*, 1994) which did not contract human pulmonary arteries without endothelium. Similar results, that is, no contraction, were observed with McN-A-343 in bovine coronary artery (Duckles, 1988). However, in arterial preparations derived from either the dog or cat (Rubanyi *et al.*, 1987; Dauphin & Hamel, 1990; Alonso *et al.*, 1991) McN-A-343 has been shown to mimic the relaxant effect of ACh, results which are similar to data obtained in human pulmonary arteries (present study).

While the results from human pulmonary arteries and other functional studies have demonstrated ACh-induced endothelium-dependent relaxations (Furchgott & Zawadzki, 1980; Angus *et al.*, 1983), several of the autoradiographic or radioligand binding studies failed to detect the presence of muscarinic receptors on endothelial cells of blood vessels (Stephenson *et al.*, 1988; Yamada *et al.*, 1990; De Michele *et al.*, 1991). In contrast, autoradiographic investigations in the rabbit thoracic aorta (Tsukahara *et al.*, 1989) and radioligand binding experiments performed in the bovine thoracic aorta (Brunner & Kukovetz, 1991; Traish *et al.*, 1994) have shown the presence of M_1 and/or M_3 receptors on endothelial cells. However, these latter results suggest that there is a small density of muscarinic receptors present on the endothelium. In addition, Tracey and Peach (1992) have shown, with northern blot analysis, mRNA transcripts encoding for the muscarinic receptors (M_1, M_2, M_3) in bovine isolated aortic endothelial cells. Thus low density as well as the differences in the radioligand used may provide an explanation as to why some investigators detected the muscarinic receptors whereas others were unable to demonstrate such sites. In the human lung, autoradiographic results (Mak & Barnes, 1989) suggested that approximately 60% of the binding to the muscarinic receptors was of the M_1 subtype. The functional data (present study) demonstrated that M_1 receptors are located on the endothelial cells in human pulmonary vessels and may partially account for this high percentage of muscarinic receptor subtype detected by autoradiographic techniques.

In tissue or cloned cells, where only the M_3 receptor is expressed, the affinity value for pirenzepine against ACh-induced response was 6.9 whereas for pFHHSiD the range was 7.8–7.9 (Eglen *et al.*, 1994). In the endothelium-removed preparations (present study), the pK_B values obtained with pirenzepine are consistent with the involvement of M_3 receptors. However, the value calculated for pFHHSiD against ACh-induced contraction was quite low, but was greater than the anomalously 'low' 7.13 obtained with M_3 receptors in tracheal preparations (Eglen *et al.*, 1990a, b). These results indicate that only M_3 receptors are associated with the contractions of vascular smooth muscle cells and are in agreement with results from several studies on animal coronary arteries which have demonstrated an M_3 receptor mediating the ACh-induced contraction (Van Charldorp *et al.*, 1988; Duckles & Garcia-Villalon, 1990; Simonsen *et al.*, 1993).

The affinity values for pirenzepine or pFHHSiD derived from ACh-induced relaxation (Table 3) were not significantly different from those expected in presence of an M_3 receptor. However, these affinity values may not exclude the presence of two muscarinic receptor subtypes on the endothelium where the density of M_3 receptors may be greater than that of M_1 receptors. Indeed, the pK_B values obtained with pirenzepine or pFHHSiD against McN-A-343 induced relaxation were the expected values for an M_1 receptor (Lambrecht *et al.*, 1988; Eltze, 1988). In addition, there was a reverse order of magnitude (Table 3) observed for the pK_B values (pirenzepine/pFHHSiD) on ACh- and McN-A-343-induced relaxations. The ACh-induced relaxation was affected by the low concentration (10 μM) of pirenzepine. Together these results suggest the presence of M_3 and M_1 receptors on the endothelium. These data derived from human pulmonary vessels are in contrast to results obtained in other vascular tissues where only M_3 receptors have been shown to be involved in the ACh-induced relaxation (Duckles & Garcia-Villalon, 1990; Jaiswal *et al.*, 1991). However, involvement of an endothelial M_1 receptor in the ACh-induced relaxation has also been suggested by Simonsen *et al.* (1993) in lamb isolated coronary arteries. Rubanyi and coworkers (1987) found in dog femoral artery

preparations, that pirenzepine was a more potent antagonist against McN-A-343-induced relaxation than against that produced by ACh. These authors as well as Hynes and coworkers (1986) showed biphasic-relaxation curves to ACh and methacholine in vascular preparations. In these experiments Rubanyi and coworkers (1987) described two components, one mediated via M_1 receptors and another by M_3 receptors. The biphasic-relaxation in human pulmonary arteries (Figure 3a) obtained with the lower concentration of pirenzepine in our study supports these observations.

The data obtained in human pulmonary arteries suggest that M_2 and M_4 receptors are not involved in the contraction or relaxation, since the affinity values calculated for the antagonist methoctramine were considerably different from the affinity values derived from experiments with tissues and cloned cells (Eglen *et al.*, 1994).

The mechanisms underlying the relaxant responses to ACh or McN-A-343 in human pulmonary arteries were cyclo-oxygenase and NO-synthase pathway dependent as has been previously described in human pulmonary arteries stimulated by histamine (Ortiz *et al.*, 1992). In the present study each inhibitor alone had only small effects in comparison with the significant inhibition obtained with the combination of both inhibitors on the ACh-induced relaxation. Other studies have shown little or no effects with these inhibitors alone on ACh-induced vasodilatations (Greenberg *et al.*, 1987; Dinh Xuan *et al.*, 1990; Crawley *et al.*, 1990). These inhibitions were not dependent on the type of muscarinic receptor stimulated, since similar results were obtained with McN-A-343. The mechanisms involved in the contractile response to ACh in human pulmonary arteries remain to be established. In endothelium-intact preparations (present study), a relaxant component was found to be involved in the ACh-induced contraction since removal of the endothelium or the addition of L-NOARG and indomethacin potentiated this contraction.

The results presented here demonstrate the role of M_1 and M_3 muscarinic receptor subtypes in the ACh-induced relaxation in human pulmonary arteries and suggest their localization on the endothelial layer. The mechanisms involved in this relaxation are dependent on the cyclo-oxygenase metabolites and NO-synthase pathway. The data indicate that the contractions induced by ACh are mediated via the activation of M_3 receptors located on the vascular smooth muscle.

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