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Evidence for a M_1 muscarinic receptor on the endothelium of human pulmonary veins

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1 To characterize the muscarinic receptors on human pulmonary veins associated with the acetylcholine (ACh)-induced relaxation, isolated venous and arterial preparations were pre-contracted with noradrenaline (10 μM) and were subsequently challenged with ACh in the absence or presence of selective muscarinic antagonists.

2 ACh relaxed venous preparations derived from human lung with a pD_2 value of 5.82 ± 0.09 ($n=16$). In venous preparations where the endothelium had been removed, the ACh relaxations were abolished ($n=4$). ACh relaxed arterial preparations with a pD_2 value of 7.06 ± 0.14 ($n=5$).

3 Atropine (1 μM), the non selective antagonist for muscarinic receptors, inhibited ACh-induced relaxations in human pulmonary veins. The affinity value (pK_B value) for atropine was: 8.64 ± 0.10 ($n=5$). The selective muscarinic antagonists (darifenacin (M_3), himbacine (M_2,M_4), methoctramine (M_2) and pFHHSiD (M_1,M_3)) also inhibited ACh-induced relaxations in venous preparations. The pK_B values obtained for these antagonists were not those predicted for the involvement of M_{2-5} receptors in the ACh-induced relaxation in human pulmonary veins.

4 The pK_B value for darifenacin (1 μM) was significantly greater in human pulmonary arterial (8.63 ± 0.14) than in venous (7.41 ± 0.20) preparations derived from three lung samples.

5 In human pulmonary veins, the pK_B values for pirenzepine (0.5 and 1 μM), a selective antagonist for M_1 receptors, were: 7.89 ± 0.24 ($n=7$) and 8.18 ± 0.22 ($n=5$), respectively. In the venous preparations, the pK_B values derived from the functional studies with all the different muscarinic antagonists used were correlated ($r=0.89$; $P=0.04$; slope $=0.78$) with the affinity values (pK_i values) previously published for human cloned m1 receptors in CHO cells.

6 These results suggest that the relaxations induced by ACh are due to the activation of M_1 receptors on endothelial cells in isolated human pulmonary veins.
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Abbreviations: ACh: acetylcholine; EC_{50}: effective concentration value; E_{max}: maximal response; K_B value: equilibrium dissociation constant; MT-1: muscarinic toxin-1; NP: not performed

Introduction

The paradoxical effects (contraction/relaxation) of acetylcholine (ACh) in vascular smooth muscle preparations are now known to be associated with the activation of receptors present on the smooth muscle as well as on the endothelial cells (Furchgott & Zawadzki, 1980; Kalsner 1989). In human isolated pulmonary arteries where tone has been induced with a contractile agent, ACh induces endothelium-dependent relaxation (Greenberg *et al*., 1987; Thom *et al*., 1987; Dinh Xuan *et al*., 1990). In contrast, either at basal tone or after removal of endothelium, ACh induces contraction in human pulmonary arteries (Norel *et al*., 1996). Walch *et al*. (1997) showed that when tone was elevated, ACh relaxed human pulmonary isolated veins, whereas at basal tone in preparations where the endothelium had been removed, pulmonary veins do not respond to this neurotransmitter. In addition, human pulmonary veins are less sensitive to the relaxant effect of ACh than arteries by a factor of 40 fold (Walch *et al*., 1997) suggesting a difference at the level of the receptors in the ACh-induced relaxation of these vessels.

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In vascular tissues, atropine prevents the vasoactive effects of ACh, suggesting that the actions of this neurotransmitter are the result of the activation of muscarinic receptors (Nandiwada *et al*., 1983; El-Kashef *et al*., 1991; El-Kashef & Catravas, 1991; Buzzard *et al*., 1993; Norel *et al*., 1996). In bioassays, the muscarinic receptors have been classified into four subtypes (M_1-M_4). However, five homologous genes, encoding for the muscarinic receptors, have been described (m1–m5; Caulfield & Birdsall, 1998). In vascular tissues, the ACh-induced contractions involve a variety of muscarinic receptors and depend on the vascular bed and the species from which the preparations are derived (Eglen *et al*., 1996). M_3 receptors are involved in the contraction of human and rabbit isolated pulmonary arteries (Norel *et al*., 1996; Altiere *et al*., 1994), whereas both M_1 and M_2 receptors are involved in the ACh-induced increases in pulmonary vascular resistance in canine pulmonary circulation (El-Kashef *et al*., 1991).

In most vascular preparations, only M_3 receptors mediate endothelium dependent relaxation (Eglen *et al*., 1996). In the rat and rabbit isolated pulmonary arteries, M_3 receptors have been implicated in the ACh-induced relaxation (McCormack *et al*., 1988; Altiere *et al*., 1994). In contrast, Norel *et al*. (1996) have shown that both M_3 and M_1 receptors mediate the ACh-

induced endothelium dependent relaxation in human isolated pulmonary arteries, suggesting that muscarinic receptor subtypes present on the pulmonary vascular endothelium may differ from the observation that activation of only M_3 subtypes mediates relaxation. Therefore, the aim of this study was to characterise the muscarinic receptor subtype(s) involved in the ACh-induced relaxation of isolated human pulmonary veins. The affinity values obtained for the different muscarinic antagonists in human pulmonary veins were compared not only with those reported in the literature but also with those obtained in isolated human pulmonary arteries.

Methods

Isolated preparations

Human lung tissue was obtained from 19 patients (16 male and three female) who had undergone lobectomy or pneumonectomy for removal of lung carcinoma. The mean age was 58 ± 2 years. Pulmonary veins and arteries (2–4 mm internal diameter) were carefully removed from the macroscopically normal regions of the diseased lung and dissected free from adjoining connective tissue and lung parenchyma. The preparations were placed in Tyrode's 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, glucose 5.5) and maintained at 4°C. All tissues were used within 1–12 h of surgery.

Vessels were cut as rings (3–5 mm in length). In some venous preparations, the endothelium 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 stretch. The rings were then set up in 10 ml organ baths containing Tyrode's solution, gassed with 5% CO_2 in O_2, at 37°C and pH 7.4. An optimal load (1.5–2 g depending on internal diameter), which ensured maximal responses to contractile agonists used, was applied to each ring. Changes in force were recorded by isometric force displacement transducer (Narco F-60) and physiographs (Linseis). Subsequently, preparations were allowed to equilibrate for 90 min with bath fluid changes every 10 min.

Experimental protocol

After the equilibration period, the vascular preparations were incubated for 30 min with Tyrode's solution (control) or with different muscarinic antagonists. These antagonists bind preferentially to the muscarinic receptors indicated in parentheses: darifenacin (M_3), himbacine (M_2, M_4), methoctramine (M_2), pFHHSiD (M_1, M_3), pirenzepine (M_1) and atropine (non-selective). Subsequently, the rings were pre-contracted with noradrenaline (10 μM). When the contraction reached a plateau, rings were stimulated with increasing concentrations of ACh (1 nM–10 mM) or muscarinic toxin-1 (MT-1; 1–100 nM), applied in a cumulative fashion. The maximal relaxation of each preparation was obtained by addition of papaverine (0.1 mM) at the end of the experimental protocol.

In some experiments, ACh stimulation was performed in vessels at basal tone, that is, the noradrenaline-induced pre-contraction was omitted.

Data analysis

The changes in force were measured from isometric recordings and expressed in grams (g). The ACh-induced relaxations were expressed either as g or as per cent of the relaxation induced with papaverine. Noradrenaline and papaverine responses were expressed as g.

The maximal response (E_{max}) produced with ACh and the effective concentration value (EC_{50}) were interpolated from the individual concentration-effect curves. The pD_2 values were calculated as the negative log of the EC_{50} values. When the pD_2 values obtained in the presence or absence of antagonist were significantly different, the equilibrium dissociation constant for the antagonist (K_B) was calculated using the following equation: $K_B = [B]/(DR-1)$. Where [B] is the concentration of the antagonist and DR (dose ratio) is the ratio of EC_{50} of agonist in the presence and absence of antagonist. The affinities of the muscarinic antagonists (pK_B) were calculated as the negative log of the K_B values.

All results are expressed as means $\pm$ s.e.mean derived from (n) different lung samples or patients. Statistical analysis was performed using Student's t-test or Student's paired t-test with a confidence level of 95%. Linear regressions were performed and the correlation coefficient (r) was calculated. Regression and significance were calculated with SigmaStat Jandel Scientific software. Slopes were calculated when the linear regressions were significant.

Compounds

The compounds and their sources were: acetylcholine chloride, noradrenaline, atropine sulphate, pirenzepine (Sigma Chemical Co., St. Louis, MO, U.S.A), pFHHSiD ((±)-p-fluorohexahydro-sila-difenidol hydrochloride), methoctramine (Research Biochemicals Inc., Natick, MA, U.S.A.), himbacine (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, U.S.A.), muscarinic toxin-1 (Alomone Labs, Ltd., Jerusalem, Israel) and papaverine hydrochloride (Meram Laboratories, 77020 Melun, France). Darifenacin hydrobromide was a generous gift from Pfizer Limited (Sandwich, Kent, U.K.).

Himbacine (0.01 M) was dissolved in 100% ethanol and subsequent dilutions were made in Tyrode solution. All other compounds mentioned above were dissolved in Tyrode's solution, each subsequent dilution was made in Tyrode's solution.

Results

During the period of incubation (30 min) with Tyrode's solution, the human pulmonary vascular isolated preparations relaxed slightly (veins: 0.19 ± 0.07 g, $n = 16$; arteries: 0.07 ± 0.11 g, $n = 5$). The muscarinic antagonists did not modify tone during this period. The noradrenaline-induced pre-contractions in the vascular preparations were (veins: 1.79 ± 0.21 g, $n = 16$; arteries: 1.26 ± 0.22 g, $n = 5$). These pre-contractions were not significantly (Student's paired t-test) modified after any incubation with a muscarinic antagonist or after removal of the endothelial layer. At the end of the protocols, papaverine (0.1 mM) relaxed the vascular preparations (veins: 2.16 ± 0.22 g, $n = 16$; arteries: 1.51 ± 0.18 g, $n = 5$).

In arterial preparations, ACh induced dose-dependent relaxations, the pD_2 value (sensitivity) was 7.06 ± 0.14 and the E_{max} was 0.95 ± 0.22 g ($n = 5$). These relaxations were significantly (Student's paired t-test) displaced in a parallel manner when arterial preparations derived from the same lung samples were incubated with darifenacin. In the presence of darifenacin (1 μM), the pD_2 value was 4.50 ± 0.15, the E_{max} was 1.33 ± 0.40 g and the pK_B value calculated from these displacements was 8.56 ± 0.15 ($n = 5$).

In venous preparations at basal tone or after noradrenaline-induced pre-contraction, ACh produced concentration-dependent relaxations (Figure 1) and the pD_2 values were not significantly different (Student's paired or unpaired *t*-test), 5.81 ± 0.16 ($n=4$) and 5.82 ± 0.09 ($n=16$), respectively. Human pulmonary veins were significantly less sensitive to ACh after noradrenaline-induced pre-contraction, when compared with human pulmonary arteries, however E_{max} were not different between these preparations (Student's unpaired *t*-test). In venous preparations pre-contracted with noradrenaline, ACh-induced relaxations were abolished after removal of endothelium $E_{max} = 0.08 \pm 0.05$ g ($n=4$). The muscarinic toxin MT-1 at the concentrations used (1–100 nM) did not relax the precontracted human pulmonary venous preparations ($n=3$).

In human pulmonary veins, pirenzepine (0.5 or 1 μM), atropine (1 μM) (Figure 2) and the other antagonists displaced the ACh concentration-dependent relaxation in a parallel manner. The significant decrease (Student's paired *t*-test) of the pD_2 values derived from these curves are presented Table 1. In addition, pK_B values were calculated from the displacement of ACh curves in human pulmonary veins and are presented in Table 1. The pK_B values obtained with darifenacin were significantly smaller in veins (Student's paired *t*-test) from those obtained above with this antagonist in paired arteries.

The pK_i values derived from binding experiments using human cloned m1–m5 receptor subtypes and the different antagonists were compiled from published reports and averaged. Correlation of the pK_B values obtained in human pulmonary veins with the average of these published pK_i values at cloned receptors are presented in Figure 3. In this Figure, the slope of the significant regression obtained with the m1 receptor subtype is indicated.

Discussion

The data obtained in human pulmonary veins (present study) suggest that ACh-induced endothelium dependent relaxation was *via* activation of muscarinic M_1 receptors. These data with muscarinic antagonists were significantly correlated with pK_i values obtained with human cloned m1 receptor.

There are few reports which characterize the endothelial muscarinic receptors in human veins. Recently, Mahdy *et al.* (1998) have suggested that muscarinic receptors are present on human hand venous endothelial cells in culture. However, only 25% of the cells were responsive to carbachol (mobilization of intracellular Ca^{2+}) and the effect of atropine was not examined. Vasodilatation mediated by endothelial muscarinic receptors was suggested in human venous vasculature in the

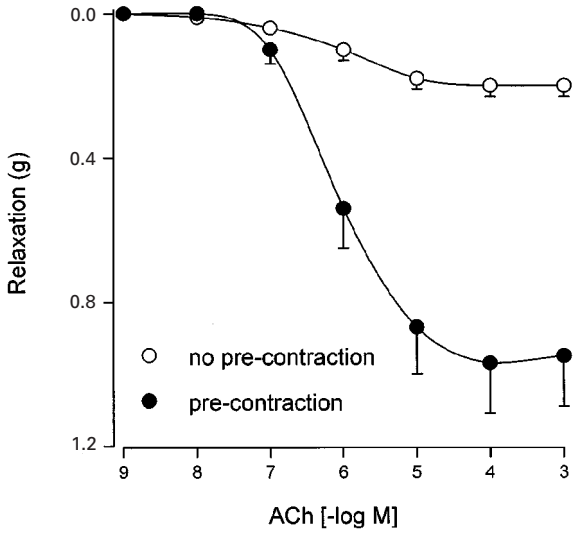

Figure 1 ACh-induced relaxation of isolated human pulmonary venous preparations at basal tone (no pre-contraction; $n=4$) or at elevated tone (noradrenaline- (10 μM) induced pre-contraction; $n=16$). Responses are expressed in (g). Data are means ± s.e.mean.

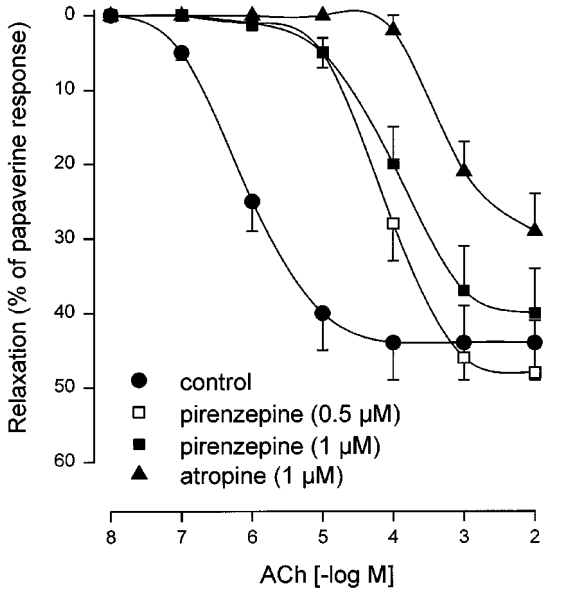

Figure 2 Effects of atropine (1 μM; $n=5$) and pirenzepine (0.5 μM; $n=5$ or 1 μM; $n=7$) on the ACh-induced relaxation of human isolated pulmonary venous preparations. Control: $n=16$. The ACh relaxations were produced after noradrenaline- (10 μM) induced pre-contractions. Responses are expressed as per cent of the relaxation induced by papaverine (0.1 mM). Values are means ± s.e.mean.

Table 1 Effect of muscarinic receptor antagonists on the relaxation induced by ACh in human pulmonary veins

Antagonist	*Concentration* (μM)	n	*pD_2 value* *Control*	*Treated*	P	*pK_B value*
Darifenacin	0.5	3	5.55 ± 0.17	3.80 ± 0.18	*	8.04 ± 0.28
	1	3	5.56 ± 0.17	4.13 ± 0.06	*	7.41 ± 0.20
Himbacine	0.5	3	5.95 ± 0.30	5.15 ± 0.23	*	7.01 ± 0.20
	1	3	5.95 ± 0.30	4.92 ± 0.30	***	6.99 ± 0.07
Methoctramine	5	4	5.75 ± 0.18	4.18 ± 0.05	**	6.85 ± 0.24
	50	4	5.61 ± 0.17	3.43 ± 0.23	***	6.48 ± 0.26
pFHHSiD	8	5	6.01 ± 0.11	3.87 ± 0.21	***	7.22 ± 0.25
Pirenzepine	0.5	5	5.83 ± 0.16	3.95 ± 0.14	***	8.18 ± 0.22
	1	7	5.83 ± 0.14	3.93 ± 0.26	***	7.89 ± 0.24
Atropine	1	5	5.84 ± 0.16	3.21 ± 0.07	***	8.64 ± 0.10

Effect of muscarinic receptor antagonists (Treated) or Tyrode's solution (Control) on ACh-induced relaxation of intact venous preparations derived from the same lung samples. The ACh relaxations were produced after noradrenaline- (10 μM) induced pre-contractions. Values are means ± s.e.mean and *n* indicates the number of lung samples used. Each pD_2 value for treated tissues was compared with the corresponding paired control value: $*P<0.05$, $**P<0.01$, $***P<0.005$ (Student's *t*-test).

forearm and in human isolated saphenous veins (Kemme *et al*., 1995; Hamilton *et al*., 1997). In contrast, ACh did not produce relaxation in human isolated epicardial veins (Saetrum Opgaard & Edvinsson, 1996). Functional studies using human isolated pulmonary veins (present report) suggest that muscarinic receptors are present in the human venous endothelium since the ACh-induced relaxations were abolished when the endothelium was removed and atropine antagonized the relaxant response.

The pK_B values obtained with the muscarinic antagonists were only significantly correlated with the pK_i values determined for the human cloned m1 receptor. Furthermore, the slope of the linear regression was near to unity. In addition, the pK_B values obtained with pirenzepine, a specific M_1 receptor antagonist (Hammer *et al*., 1980; Brown *et al*., 1980), were similar to those reported for human hippocampus (7.6) and for human vas deferens (7.4), which are human tissues containing predominantly M_1 receptor subtypes (Gies *et al*., 1989; Miranda *et al*., 1992). In contrast, the affinity value for darifenacin (preferential M_3 antagonist) derived from ACh-induced relaxation in veins (7.73; present report) did not provide evidence in support of the presence of an M_3 receptor. A greater affinity value for darifenacin was found in human pulmonary artery (8.56; present report) where M_3 receptors have been described (Norel *et al*., 1996). The pK_i value expected for this antagonist in CHO cells expressing only m3 receptors was reported to be 8.75 (Figure 3) and the pA_2 values obtained in some bioassays were higher (guinea-pig oesophageal muscularis mucosae, ileum or trachea, 9.1–9.5, Eglen *et al*., 1996; rabbit iris sphincter 9.42 and urinary bladder 9.09, Choppin *et al*., 1998). In addition, pFHHSiD (preferential M_1/M_3 antagonist) exhibits a pK_B value, in human pulmonary veins, closer to the value for M_1 receptors than for M_3 receptors (Figure 3). The affinity values obtained (Table 1) with himbacine (preferential M_2/M_4 antagonist) and methoctramine (preferential M_2 antagonist) were not in agreement with the presence of M_2 or M_4 receptors. These affinity values were not in agreement with data obtained from binding experiments (Figure 3) or from the classical bioassays. In bioassays for M_2 receptors (guinea-pig or rat atria; Eglen & Harris, 1993; Lazareno *et al*., 1990) or for M_4 receptors (chicken lung or rabbit lung; Lazareno *et al*., 1990), the affinity values were reported for these antagonists to be between (7.8–8.3).

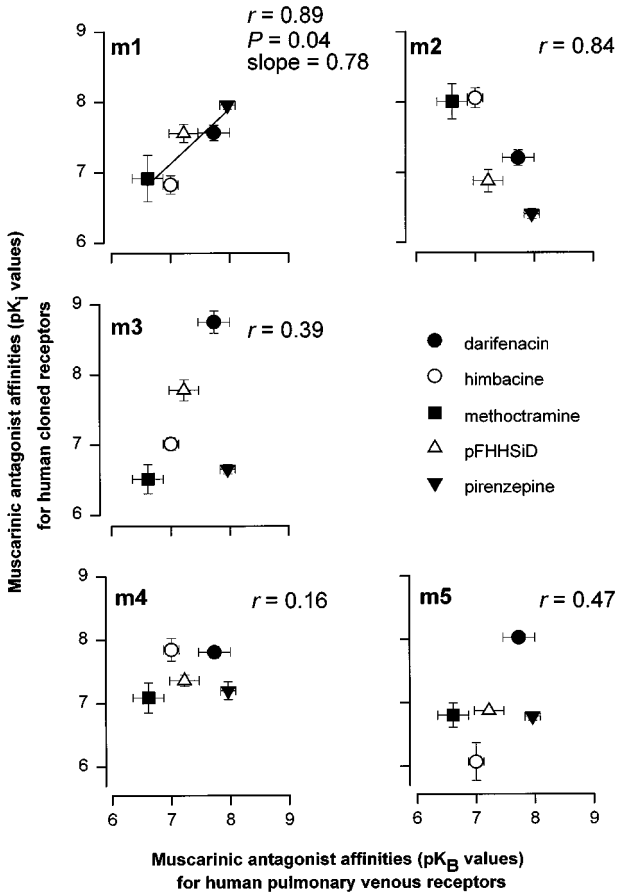

Figure 3 Correlation between the affinity values of muscarinic receptor antagonists on the relaxation induced by acetylcholine in human pulmonary veins (pK_B values, shown in Table 1) and the average of the affinity values of muscarinic receptor antagonists for human cloned m1-m5 receptor subtypes (pK_i values, obtained from Bolden *et al*., 1992; Buckley *et al*., 1989; Cembala *et al*., 1998; Dörje *et al*., 1991; Eglen *et al*., 1996; Gillberg *et al*., 1998; Jolkkonen *et al*., 1994; Miller *et al*., 1992; Nelson *et al*., 1995; Nunn *et al*., 1996; Rinken, 1995). The pK_B values obtained with one antagonist at different concentrations (darifenacin, 0.5 and 1 μM; himbacine, 0.5 and 1 μM; methoctramine, 5 and 50 μM; pirenzepine, 0.5 and 1 μM) for each lung sample were averaged since values were not statistically different (Student's *t*-test or Student's paired *t*-test).

Kornisiuk *et al*. (1995) have shown that the muscarinic toxin MT-1 from the venom of the green mamba (Dendroaspis angusticeps) inhibits the N-[^{3}H]-methylscopolamine binding with high affinity on the human m1 (K_i = 48 nM) and on the human m3 (K_i = 72 nM) cloned receptors. This toxin did not affect the binding on human m2 and m4 cloned receptors. Jolkkonen *et al*. (1995) have reported that MT-1 produced contraction in the guinea-pig ileum, suggesting that this toxin may be a muscarinic agonist. In human pulmonary veins (present report), challenge with increasing concentrations of MT-1 did not produce relaxation. Since there are very few functional studies using MT-1, the agonist potency of MT-1 remains to be established.

There are few reports using venous tissues to examine the muscarinic receptor subtypes involved in the ACh-induced relaxation. A study performed on rabbit external jugular vein suggests that M_1 and/or M_3 receptors are involved in the ACh-induced relaxation (Martin *et al*., 1992). In contrast, the muscarinic receptor subtypes implicated in the ACh-induced vasodilatation were extensively studied in arteries. Several studies have shown that the ACh-induced relaxations in these vessels were mediated by the M_3 receptor subtype localized on the endothelium (rabbit aorta: Jaiswal *et al*., 1991; rabbit ear artery: Duckles & Garcia-Villalon, 1990; cat cerebral artery: Dauphin & Hamel, 1990; bovine coronary artery: Brunner *et al*., 1991; simian coronary arteries: Ren *et al*., 1993; rat pulmonary artery: McCormack *et al*., 1988, rabbit pulmonary artery: Altiere *et al*., 1994). Similar results were obtained *in vivo* in the human forearm vasculature (Bruning *et al*., 1995). However, there is some evidence to suggest that M_1 receptors may also be present in endothelial cells. In fact, M_1 and M_3 receptors are involved in the ACh-induced relaxation in canine isolated coronary artery and in human isolated pulmonary artery (Rubanyi *et al*., 1987; Norel *et al*., 1996). M_1 and M_3 receptors were also characterised using radioligand binding techniques in human and bovine cerebral capillary membranes which were principally derived from endothelial cells (Linville & Hamel, 1995). Finally, Simonsen *et al*. (1993; 1997) have shown that endothelial M_1 receptors modulate the ACh-induced contraction of lamb isolated coronary small arteries.

At basal tone, human isolated pulmonary arteries contract in presence of ACh and this response is amplified when endothelium is removed (Norel *et al*., 1996). In this latter

report, the receptor associated with the ACh-induced contraction in pulmonary vascular smooth muscle was an M_3 subtype. At basal tone, human pulmonary isolated veins slightly relaxed in presence of ACh (present report) and when the endothelium was removed, veins were not responsive to ACh (Walch *et al*., 1997). These results suggested that in smooth muscle of human pulmonary veins, in contrast to arteries, there are no muscarinic receptors present since the tissues do not contract. However, the ability of ACh to induce pulmonary venous contraction is species dependent since this mediator induced contraction in canine, goat and sheep isolated pulmonary veins (Furuta *et al*., 1987; Chand, 1981; Toga *et al*., 1996). In these latter studies, the receptors implicated in the ACh-induced contraction in pulmonary veins were not identified. Nevertheless, the involvement of M_1 receptors was suggested in the ACh-induced contraction of canine saphenous vein (O'Rourke & Vanhoutte, 1987; Eglen *et al*., 1996).

The results presented in this report confirm the absence of contraction induced by ACh in human pulmonary veins. In addition, they demonstrate the role of an endothelial M_1 muscarinic receptor subtype in the ACh-induced relaxation of the human pulmonary veins. These results are in contrast with the presence of two receptors (M_1 and M_3) described for the ACh relaxations observed in human pulmonary artery (Norel *et al*., 1996). This discrepancy may explain the difference of sensitivity observed in these vessels during ACh-induced relaxations (Walch *et al*., 1997).

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