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Role of mitogen-activated protein kinase pathway in acetylcholine-mediated *in vitro* relaxation of rat pulmonary artery

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Abstract

This study was designed to characterise the muscarinic receptor subtype responsible for acetylcholine-mediated *in vitro* pulmonary artery relaxation in rats and the importance of the presence of neostigmine (an anti-cholinesterase) during receptor characterisation. Cumulative administration of acetylcholine elicited concentration-dependent relaxation of phenylephrine (1 μM) precontracted preparations. Inclusion of neostigmine (10 μM) caused a parallel leftward shift with an increase of the pD_2 value (7.09 vs. 6.43) of the concentration–response curve of acetylcholine. The magnitude of maximum relaxation, however, was not affected. Using a range of conventional muscarinic receptor antagonists (atropine, pirenzepine, methoctramine, *p*-FHHSiD and tropicamide) and the highly selective Green Mamba muscarinic toxins (MT-3 and MT-7), it was found that muscarinic M_3 receptors are probably responsible for endothelium-dependent relaxation of the pulmonary artery upon acetylcholine challenge. Preincubation with N^G-nitro-L-arginine methyl ester (L-NAME, 20 μM, a nitric oxide synthase inhibitor), but not N^G-nitro-D-arginine methyl ester (D-NAME, 20 μM), abolished acetylcholine-elicited relaxation. Moreover, 6-anilino-5,8-quinolinedione (LY 83583, 1 μM) and methylene blue (1 μM) (both are guanylate cyclase inhibitors) markedly attenuated acetylcholine-elicited relaxation. However, the presence of indomethacin (3 μM, a cyclo-oxygenase inhibitor), (−)-perillic acid (30 μM, a $p21^{ras}$ blocker), 2-[2′-amino-3′-methoxy-phenyl]-oxana-phthalen-4-one (PD 98059) (10 μM, a p42/p44 mitogen-activated protein kinase inhibitor), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB 203580) (1 μM, a p38 mitogen-activated protein kinase blocker), wortmannin (500 nM, a phosphatidylinositol-3 kinase inhibitor) and genistein (10 μM, a tyrosine kinase blocker) failed to alter acetylcholine-provoked pulmonary arterial relaxation. These results suggest that acetylcholine caused pulmonary arterial relaxation through the activation of muscarinic M_3 receptors in the endothelium. Moreover, the $p21^{ras}$/mitogen-activated protein kinase pathway seems to play no role in mediating acetylcholine-elicited relaxation. © 2002 Published by Elsevier Science B.V.

Keywords: Acetylcholine; Pulmonary artery, rat; Muscarinic M_3 receptor; Nitric oxide (NO); MAP (mitogen-activated protein) kinase

1. Introduction

Blood flow in the pulmonary circulation, as in the rest of the body, is under the control of the autonomic nervous system (sympathetic and parasympathetic systems). The sympathetic nervous system mainly mediates vasoconstriction through the activation of noradrenaline on α-adrenoceptors whereas acetylcholine released from parasympathetic nerves contributes to the biphasic response in the pulmonary circulation. The biphasic response to acetylcholine in the pulmonary vascular bed is dependent on the vascular tone. Under resting tone, acetylcholine induces vasoconstriction whereas, with a raised tone, it causes vasodilatation (Nandiwada et al., 1983; El-Kashef and Catravas, 1986; Matran et al., 1991).

It has been well documented that relaxation of the pulmonary artery in response to acetylcholine is the result of activation of the muscarinic receptors located on the endo-

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thelium (Nandiwada et al., 1983; El-Kashef et al., 1991; Norel et al., 1996). Up to now, five subtypes of muscarinic receptor (m1 to m5), coded by five homologous genes have been identified by molecular cloning techniques (Caulfield and Birdsall, 1998). Only four subtypes (M_1 to M_4), however, can be differentiated pharmacologically using relatively selective muscarinic receptor antagonists. Most evidence suggests that receptor subtypes identified through pharmacological methods corresponds to the like-numbered molecular form (e.g. M_3 = m3). It was found that in most vascular preparations, including rat and rabbit isolated pulmonary arteries, the endothelium-dependent relaxation is mediated by muscarinic M_3 receptors (McCormack et al., 1988; Altiere et al., 1994; Eglen et al., 1996). However, both muscarinic M_1 and M_3 receptors were reported involving in the acetylcholine-induced vasorelaxation in human isolated pulmonary artery (Norel et al., 1996). This suggests that activation of the muscarinic M_3 subtypes of the muscarinic receptors might not be the only muscarinic receptor mediating the endothelium-dependent relaxation in the pulmonary vasculature upon acetylcholine challenge.

Most previous studies that had relied on competitive receptor antagonism for characterisation of muscarinic receptor subtype(s) using acetylcholine did not include any cholinesterase inhibitor (Duckles, 1988; Hohlfeld et al., 1990; El-Kashef and Catravas, 1991; Jaiswal et al., 1991; Norel et al., 1996; Laurence et al., 2000), which could pose a problem. The continuous breakdown of acetylcholine by cholinesterase (El-Bermani et al., 1982) creates a nonequilibrium condition during the experiment. This can alter the shape and/or slope of the Schild plot, implying that the estimated pA_2 values as well as the conclusions about the subtype of muscarinic receptor may not be accurate. Besides, it has been suggested that abnormal parasympathetic activity may be responsible for the development of some pulmonary vascular diseases (Dinh Xuan et al., 1989; Calver et al., 1992). Hence, there is a need for recharacterisation of the muscarinic receptor subtype(s) involved in the acetylcholine-mediated endothelium-dependent vasorelaxation of the pulmonary artery. Therefore, the first objective of this study was to demonstrate the importance of including an anti-cholinesterase for characterisation of the muscarinic receptor subtype involved in acetylcholine-induced pulmonary artery relaxation.

It is well established that vasodilatation induced by acetylcholine is dependent on the endothelium and is mediated by the endothelium-derived relaxing factor, which was later identified as nitric oxide (Furchgott and Zawadzki, 1980; Gruetter et al., 1979; Palmer et al., 1987). In addition to nitric oxide, prostanoids (in particular, prostacyclin and thromboxane A_2) and endothelium-derived hyperpolarizing factor (Rang et al., 1999) are also suggested to be important in controlling vascular tone. In the present study, we also investigated the role of prostanoids (as well as endothelium-derived hyperpolarizing factor) in acetylcholine-mediated pulmonary arterial vasodilatation.

Since the discovery of the role of the endothelium-derived relaxing factor in mediating acetylcholine-induced vascular relaxation, there has been increasing interest in delineating the underlying intracellular signalling pathway mediating vasodilatation triggered by acetylcholine. Recent findings have suggested that the acetylcholine-stimulated production of nitric oxide is, in fact, more complicated than originally anticipated. Both phosphatidylinositol 3-kinase (Auger et al., 1989) and tyrosine kinase have been demonstrated to mediate, at least in part, nitric oxide production of rat basilar arterial endothelial cells in response to acetylcholine challenge (Kitazono et al., 1998; Kitayama et al., 2000). Besides producing vasodilatation, nitric oxide can also stimulate the nuclear translocation of transcription factors (Lander et al., 1996) and the downstream nitric oxide-signalling pathway involves low molecular weight G proteins like $p21^{ras}$ and mitogen-activated protein kinases. Activation of these mitogen-activated protein kinase pathways has been shown to play an important role in the tone development of vascular tissues on challenge by some neurotransmitters. Hence, it was of interest to investigate whether phosphatidylinositol 3-kinase, tyrosine kinase and mitogen-activated protein kinase pathways are also involved in acetylcholine-mediated relaxation of rat pulmonary artery.

2. Materials and methods

2.1. Tissue preparation

Sprague–Dawley rats (male, 250–350 g) were killed by cervical dislocation. The pulmonary artery (1st branch) was isolated, cleared of adhering fat and connective tissue, and was then cut into ring segments $\sim$ 2 mm in length. To study the involvement of endothelium in relaxation, some tissues had the endothelium carefully removed by gentle rubbing of the intima of the vessels with a wooden toothpick. Each ring segment was mounted on two stainless-steel hooks in an organ bath containing prewarmed (37 ± 1 °C) and oxygenated (95% O_2/5% CO_2) modified Krebs' solution. The modified Krebs' solution contained (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11, $CaCl_2$ 2.5. Before the start of experiment, the preparations were allowed to equilibrate for 60 min under a resting tension of 10 ± 0.5 mN. The bath fluid was changed every 15 min during the equilibration period. As response, the change in isometric tension was measured with a force-displacement transducer (Grass, FT-03) coupled to a MacLab Data Acquisition System. The Animal Ethics Research Committee of the Chinese University of Hong Kong approved the experiments performed in this study (approval number: 00/016/MIS).

2.2. Experimental protocols

After equilibration, the preparations were sensitised with 40 mM $[K^+]_o$ until two consecutive responses to $[K^+]_o$

were reproducible. Then acetylcholine (10 μM) was added to induce relaxation (>60%) of the preparations precontracted with noradrenaline (1 μM) for confirmation of endothelial integrity. Subsequently, preparations were incubated with 10 μM neostigmine (as control) for 30 min before precontraction with phenylephrine (1 μM). Phenylephrine 1 μM gave rise to 90% of the contractile response triggered by 50 mM $[K^+]_o$. In the preliminary study, 50 mM $[K^+]_o$ elicited maximum contraction of the pulmonary artery and was used as reference for pulmonary artery contraction. To characterise the muscarinic acetylcholine receptor subtypes and to explore the intracellular pathway mediating relaxation, a variety of selective receptor antagonists or inhibitors were incubated together with neostigmine for 30 min. Where stated, the concentration of antagonists/blockers used in this study was the effective concentration of individual blocker previously reported as affecting the "particular site" of the intracellular pathway. Experiments were repeated with different concentrations of individual muscarinic receptor antagonists, and only one concentration–response curve was made with each tissue preparation. After the precontraction tone reached steady state, a concentration–response curve was made by cumulative addition of acetylcholine or McN-A-343 to the organ bath at 5-min intervals.

2.3. Data analysis

Relaxation in response to the cholinergic receptor agonists was expressed as percentage of the phenylephrine-induced tone, and 100% relaxation was accepted when tension returned to its baseline level. Log agonist concentration–response curves in the absence (control) and the presence of an antagonist/inhibitor were generated using Prism (ver. 3.02) (GraphPad, USA), and the log EC_{50} values of the two curves were determined, where EC_{50} refers to the concentration of the agonist giving rise to 50% maximum relaxation.

In the present study, three to six concentrations of individual muscarinic receptor antagonist were used to determine the antagonist affinity, using Schild plot analysis. For each concentration of muscarinic receptor antagonist, the dose ratio (DR) was calculated using the equation: $\log (DR) = \log EC_{50(antagonist)} - \log EC_{50(control)}$. Schild plots of $\log (DR - 1)$ versus log antagonist concentration ([antagonist]) were constructed according to the equation, $\log (DR - 1) = \log [antagonist] + pA_2$. pA_2, a measure of the antagonist affinity, is defined as the negative logarithm of the antagonist concentration that produces a two-fold rightward shift in the concentration–response curve. The pA_2 values were estimated from the *x*-intercepts of Schild plots analysed by linear regression. The data were expressed as means ± S.E.M. ($n = 6–9$ except in the experiments using snake venom toxins (MT-3 and MT-7) with which $n = 4–5$ for each concentration of individual toxin tested) and comparisons between means were made using one-way analysis of variance (ANOVA). Differences between means were considered significant when $P < 0.05$.

2.4. Drugs

Chemicals used in this study included: acetylcholine chloride, atropine sulphate, carbamylcholine chloride (carbachol), N^G-nitro-D-arginine methyl ester (D-NAME), indomethacin, N^G-nitro-L-arginine methyl ester (L-NAME), methylene blue and neostigmine bromide (all were purchased from Sigma-Aldrich, USA). Pirenzepine dihydrochloride, tropicamide, methoctramine tetrahydrochloride, *p*-fluoro-hexa-hydro-sila-difenidol (*p*-FHHSiD), 6-anilino-5,8-quinolinedione (LY 83583) and 4-(3-chlorophenylcarbamoyloxy)2-butynyltrimethylammonium chloride (McN-A-343) were obtained from Research Biochemicals, USA. Muscarinic toxin-7 (MT-7, *Dendroaspis angusticeps*) was purchased from Peptide Institute (Japan) and muscarinic toxin-3 (MT-3, *D. angusticeps*) was obtained from Alomone Labs. (Israel). 2-[2′ -amino-3′ -methoxy-phenyl]-oxana-phthalen-4-one (PD 98059), (−)-perillic acid, genistein, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB 203580) and wortmannin were purchased from Calbiochem-Novabiochem, USA. Indomethacin, (−)-perillic acid, PD 98059, SB 203580 and LY 83583 were dissolved in dimethyl sulfoxide. Stock solutions of other drugs were prepared in deionized water. Aliquots of stock solutions were kept frozen until use.

3. Results

Acetylcholine produced a concentration-dependent relaxation of the pulmonary arteries precontracted with phenylephrine (1 μM) over the concentration range of 1 nM to 3 μM (Fig. 1). Relaxation was reversed into contraction at higher concentrations (>3 μM) (data not shown).

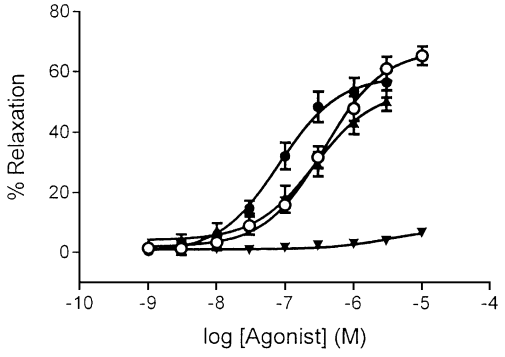

Fig. 1. Cumulative concentration–response curves for acetylcholine-mediated relaxation of phenylephrine (1 μM) precontracted pulmonary artery in the absence (○, control) and the presence of neostigmine (●) (10 μM). For comparison, relaxation–response curves of carbachol (a nonhydrolyzable analogue of acetylcholine) (▲) and McN-A-343 (▼) were included. Results are expressed as means ± S.E.M., $n = 6–9$.

3.1. Effect of neostigmine on the concentration–response curve for acetylcholine-induced relaxation of pulmonary artery

To investigate the effect of an anti-cholinesterase, acetylcholine concentration–response curves were made in the absence and presence of neostigmine (10 μM) and compared. The maximal relaxation response to acetylcholine was not significantly altered in the presence of neostigmine (Fig. 1). However, there was a significant leftward shift of the acetylcholine concentration–response curve after the addition of neostigmine (Fig. 1). The pD_2 values of the concentration–response curves of acetylcholine with and without neostigmine are 7.09 and 6.44, respectively ($P < 0.05$). In endothelium-denuded preparations, neostigmine (10 μM) markedly enhanced acetylcholine-elicited contraction of the pulmonary artery under resting tension ($n = 8$) (not shown). In view of the importance of neostigmine, pulmonary arterial rings were incubated with neostigmine (10 μM) for 30 min before acetylcholine concentration–response curves were made.

3.2. Vasorelaxing effect of carbachol

Carbachol (carbamycholine chloride) is the compound formed when a carbamyl group replaces the acetyl group of acetylcholine. The presence of the carbamyl group confers resistance of carbachol to degradation by acetylcholinesterase. Carbachol is therefore generally used as the nonhydrolyzable analogue of acetylcholine. To examine whether carbachol can mimic the effect of acetylcholine (in the presence of neostigmine), experiments were repeated using carbachol. Fig. 1 showed that the concentration–response curve of carbachol was not entirely the same as that of acetylcholine. A

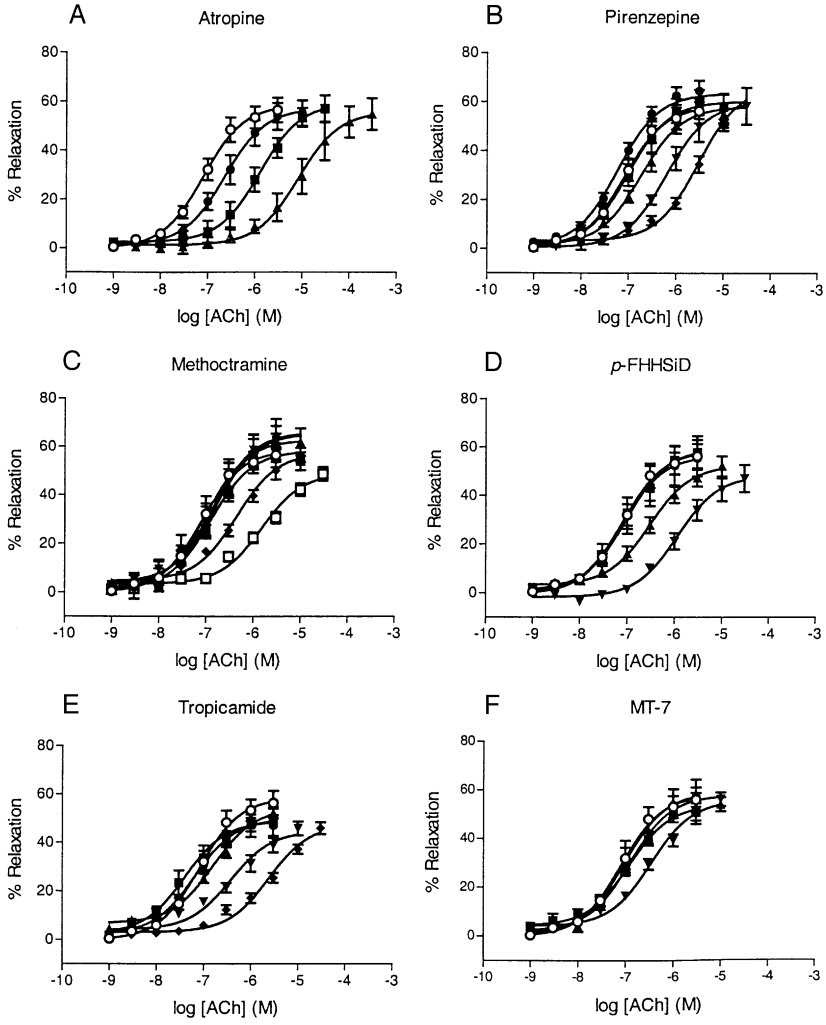

Fig. 2. Cumulative concentration–response curves for acetylcholine (○, control with 10 μM neostigmine) in phenylephrine (1 μM) precontracted pulmonary artery in the presence of: (A) atropine (●, 1 nM; ■, 10 nM; ▲, 100 nM); (B) pirenzepine (●, 1 nM; ■, 10 nM; ▲, 100 nM; ▼, 1 μM; ♦, 10 μM); (C) methoctramine (●, 1 nM; ■, 10 nM; ▲, 100 nM; ▼, 1 μM; ♦, 3 μM; □, 10 μM); (D) *p*-FHHSiD (●, 1 nM; ■, 10 nM; ▲, 100 nM; ▼, 1 μM); (E) tropicamide (●, 1 nM; ■, 10 nM; ▲, 100 nM; ▼, 1 μM; ♦, 10 μM); and (F) muscarinic toxin MT-7 (■, 1 nM; ▲, 3 nM, ♦, 10 nM; ▼, 30 nM). Results are expressed as means ± S.E.M., $n = 6–9$ except for MT-7 experiment where $n = 4–5$.

Table 1
Estimated pA_2 values of different muscarinic receptor antagonists against acetylcholine-mediated pulmonary artery (endothelium intact) relaxation (in the presence of 10 μM neostigmine) of the Sprague–Dawley rats

Antagonist	pA_2	r^2	Slope of Schild plot
Atropine	9.37	0.98	0.87 ± 0.10
Pirenzepine	7.10	0.91	0.74 ± 0.01[a]
Methoctramine	6.44	0.90	0.70 ± 0.05[a]
p-FHHSiD	7.37	0.96	0.87 ± 0.13
Tropicamide	6.92	0.91	0.77 ± 0.09[a]
MT-3	–	–	–
MT-7	–	–	–

Muscarinic toxins MT-3 and MT-7 (1, 3, 10 and 30 nM) had no apparent effect on acetylcholine-induced relaxation of phenylephrine (1 μM) precontracted pulmonary artery. Hence, pA_2 values of these compounds could not be determined.

[a] $P < 0.05$ indicates significant deviation from unity.

slightly smaller relaxant response was recorded in the presence of 100 and 300 nM carbachol (relaxation: 15.3 ± 3.2% and 31.4 ± 3.3%, respectively) compared to the response elicited by the same concentrations of acetylcholine (relaxation: 100 nM: 33.2 ± 4.4%; 300 nM: 46.4 ± 5.3%) ($P < 0.05$). Moreover, the presence of neostigmine (10 μM) did not modify the carbachol-elicited pulmonary arterial relaxation ($n = 6$) (not shown).

3.3. Identification of the subtype(s) of muscarinic receptor

Atropine (1, 10, 100 nM), a nonselective muscarinic receptor antagonist, shifted the acetylcholine concentration–response curve to the right in a parallel manner with no apparent change in the magnitude of maximum relaxation (Fig. 2). A pA_2 value of 9.37 was estimated for atropine from the Schild regression analysis. Moreover, the estimated slope of the Schild plot was not significantly deviated from unity (Table 1).

Pirenzepine (1, 10, and 100 nM; 1 and 10 μM), a selective muscarinic M_1 receptor antagonist (Hammer and Giachetti, 1982), caused a rightward parallel displacement of the curve only at concentrations of 100 nM and 1 and 10 μM (Fig. 2). With all concentrations of pirenzepine except 10 μM, there were no significant changes in the magnitude of the maximum relaxation. A pA_2 value of 7.1 was obtained and unlike the atropine slope of the Schild plot, was different from unity (Table 1).

Similarly to pirenzepine, higher concentrations of *p*-fluoro-hexa-hydro-sila-difenidol (*p*-FHHSiD) (100 nM and 1 μM), a selective muscarinic M_3 receptor antagonist (Buckley et al., 1989), produced a significant parallel rightward shift with no significant change in extent of the maximum relaxation (Fig. 2). A pA_2 value of 7.37 was estimated, with the slope of Schild plot close to unity (Table 1). On the other hand, methoctramine (1, 10 and 100 nM; 1 μM) and tropicamide (1, 10, and 100 nM), selective muscarinic M_2 and M_4 receptor antagonists, respectively (Buckley et al., 1989; Jaiswal and Malik, 1991), showed no effect on the relaxation response to acetylcholine. A higher concentration of methoctramine (3 and 10 μM) and tropicamide (1 and 10 μM) caused a nonparallel rightward shift with suppression of acetylcholine-elicited relaxation. A pA_2 value of 6.44 and 6.92 was estimated for methoctramine and tropicamide, respectively (Table 1), with the slope of Schild plot deviating significantly from unity (Table 1). Neither muscarinic toxin-3 (MT-3) (1, 3, 10 and 30 nM) (a highly selective muscarinic M_4 receptor antagonist) (Jerusalinsky et al., 2000) nor muscarinic toxin-7 (MT-7) (1, 3, 10 and 30 nM) (a highly selective muscarinic M_1 receptor antagonist) (Olianas et al., 2000) modulated significantly the acetylcholine-mediated relaxation (Fig. 2).

The effect of 4-(3-chlorophenylcarbamoyloxy)2-butynyltrimethylammonium chloride (McN-A-343; a selective muscarinic M_1 receptor agonist) (Eglen et al., 1987) was also determined. In marked contrast to acetylcholine, McN-A-343 did not induce relaxation of the pulmonary artery and no apparent change in precontracted tone was observed with concentrations < 3 μM (Fig. 1). None of the conventional muscarinic receptor antagonists and the snake venom toxins (MT-3 and MT-7) used in the study caused any significant effect on the resting tone of the pulmonary artery during incubation.

3.4. Role of endothelium

The dependence of acetylcholine-elicited relaxation on the presence of a functional endothelium was verified by repeating the experiment in preparations without endothelium. Mechanical removal of the endothelium abolished acetylcholine-mediated pulmonary artery relaxation. Application of acetylcholine produced no change in the precontracted vascular tone and sometimes induced further contraction. The maximum relaxation with acetylcholine (3 μM) decreased from 58.5 ± 2.8% in control to 4.1 ± 1.8% after the removal of endothelium (Fig. 3) ($P < 0.001$).

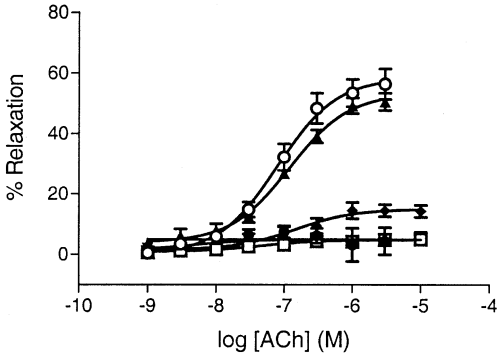

Fig. 3. Cumulative concentration–response curve for acetylcholine (○, control with 10 μM neostigmine) in phenylephrine (1 μM) precontracted pulmonary artery with intact endothelium (○), with endothelium removed (●) and in the presence of L-NAME (□, 20 μM), D-NAME (▲, 20 μM), LY 83583 (▼, 1 μM) and methylene blue (♦, 1 μM). Results are expressed as means ± S.E.M., $n = 6–9$.

3.5. Verification of the nitric oxide-guanylate cyclase pathway in acetylcholine-induced relaxation of pulmonary artery

In our preliminary study, the application of a single concentration of N^G-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor) (20 μM) into the organ bath caused a progressive increase in resting tone of the pulmonary artery ($n=5$). However, L-NAME added as three separate doses (8, 8 and 6 μM) at 5- to 7-min intervals only caused a minimal increase of the resting tone ($\sim$4% of 50 mM $[K^+]_o$-induced contraction). It was therefore decided to add L-NAME (20 μM) the same way. L-NAME (20 μM) abolished acetylcholine-mediated relaxation of the pulmonary artery (Fig. 3). On the other hand, application of N^G-nitro-D-arginine methyl ester (D-NAME) (20 μM, an inactive analogue of L-NAME) failed to modulate acetylcholine-induced pulmonary artery relaxation.

Preincubation of pulmonary artery with the soluble guanylate cyclase inhibitors, methylene blue (1 μM) and 6-anilino-5,8-quinolinedione (LY 83583) (1 μM), markedly suppressed acetylcholine-mediated pulmonary artery relaxation. A greater inhibitory effect was observed with LY 83583 than with methylene blue (Fig. 3). Moreover, LY 83583 (1 μM) but not methylene blue (1 μM) caused an increase of the resting tone ($\sim$6% of 50 mM $[K^+]_o$-induced contraction) of the pulmonary artery (data not shown).

3.6. Role of prostanoids on vasorelaxation induced by acetylcholine

To evaluate the participation of cyclo-oxygenase products on acetylcholine-induced relaxation of pulmonary artery, experiments were performed in the presence of indomethacin (1 and 3 μM, a nonselective cyclo-oxygenase inhibitor). With indomethacin, the relaxation response of the pulmonary artery preparation to acetylcholine challenge was not affected ($n=6$ for each concentration of indomethacin). The addition of indomethacin had no effect on the resting tone of the arterial preparation (not shown).

3.7. Effect of tyrosine kinase and phosphatidylinositol-3 kinase activation on acetylcholine-induced relaxation of the pulmonary artery

Preincubation of the pulmonary arterial preparations with genistein (3 and 10 μM, a tyrosine kinase inhibitor) and wortmannin (100 and 500 nM, a phosphatidylinositol-3 kinase blocker) failed to modify the acetylcholine-elicited relaxation response ($n=5-7$ for each concentration of agent tested). A higher concentration of genistein (30 μM) was also tested and it caused a marked suppression of the phenylephrine-induced tone ($n=5$). It was therefore decided not to test genistein at such a high concentration (data not shown).

3.8. Role of p21ras and mitogen-activated protein kinase activation in acetylcholine-mediated relaxation of the pulmonary artery

Similarly to genistein and wortmannin, neither (−)-perillic acid (30 μM, a selective inhibitor of $p21^{ras}$) nor 2-[2′-amino-3′-methoxy-phenyl]-oxana-phthalen-4-one (PD 98059) (10 μM, a p42/p44 mitogen-activated protein kinase inhibitor) altered acetylcholine-mediated relaxation of the pulmonary artery. 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB 203580) (1 μM, a p38 mitogen-activated protein kinase inhibitor) also failed to modify acetylcholine-elicited pulmonary artery relaxation (not shown). A higher concentration of (−)-perillic acid (100 μM) ($n=6$), PD 98059 (30 μM) ($n=6$) and SB 203580 (3 μM) ($n=6$) was not used because the magnitude of phenylephrine-induced contraction was suppressed and the induced tone was not maintained.

4. Discussion

The adult pulmonary circulation is a low-pressure, low-resistance system. Acetylcholine, the neurotransmitter of the parasympathetic nervous system, plays a role in regulating vascular tone. Acetylcholine evokes a characteristic biphasic response (contraction and relaxation) in the pulmonary vascular bed and relaxation occurs when vascular resistance is increased.

It is well known that the relaxation response produced by acetylcholine is mediated through activation of muscarinic receptors, as evidenced by the competitive antagonism seen when different muscarinic antagonists are used (Nandiwada et al., 1983; El-Kashef et al., 1991; Norel et al., 1996). In our study, atropine (a nonselective muscarinic receptor antagonist) caused a parallel rightward shift of the acetylcholine concentration–response curve and yielded a Schild plot with slope close to unity. These results indicated that muscarinic receptors are, indeed, involved in acetylcholine-induced relaxation of the phenylephrine precontracted rat pulmonary artery.

It was noted that most studies (both *in vitro* and *in vivo*) reported upon in the literature (Duckles, 1988; McCormack et al., 1988; Eglen et al., 1990; Hohlfeld et al., 1990; El-Kashef and Catravas, 1991; Jaiswal and Malik, 1991; Jaiswal et al., 1991; El-Kashef et al., 1991; Norel et al., 1996; Hoover and Neely, 1997) and characterising the subtype(s) of muscarinic receptor involved in the vascular responses to acetylcholine did not include any anti-cholinesterase during the experiments. However, it has been shown that acetylcholinesterases are present in the pulmonary artery (Altiere et al., 1994). During experiments, the enzyme's catabolic activity may pose a problem regarding the identification of the subtype of muscarinic receptor involved. It is because continuous degradation of acetylcholine by cholinesterase at the receptor site can reduce the concentration of acetylcholine

more than anticipated. Hence, results obtained from these studies may be questionable as it was relied on competitive antagonism using a range of conventional muscarinic receptor antagonists. This, therefore, prompted us to recharacterise the muscarinic receptor subtype(s) responsible for acetylcholine-induced relaxation of rat pulmonary artery in the presence of neostigmine (a cholinesterase inhibitor).

The importance of the presence of an anticholinesterase was made clear by our experimental results, which showed an ''improvement'' of the pD_2 values from 7.09 to 6.44 (a factor of 4.5) after the addition of an acetylcholinesterase inhibitor, neostigmine. Hence, neostigmine (10 μM) was incubated with the arterial preparations in all subsequent experiments. One could suggest that carbachol, which contains a carbamyl group making it resistant to degradation by acetylcholinesterase, can replace or mimic acetylcholine in studies on the effects of acetylcholine in various tissues. This issue was also addressed in our study by comparing the relaxant responses elicited by acetylcholine and by carbachol. Our results did reveal a difference between the magnitude of relaxation responses triggered by these muscarinic receptor agonists (acetylcholine (plus neostigmine) and carbachol) at the same concentration. The underlying reason(s) for the discrepancy is not known. Nevertheless, it suggested that carbachol ''might not'' truly represent or mimic the endogenous neurotransmitter, acetylcholine (with neostigmine) in producing relaxation responses in the pulmonary arteries.

It has been suggested that the muscarinic receptor subtype responsible for the relaxation responses was of the muscarinic M_3 receptor type in rat pulmonary artery (McCormack et al., 1988) and rabbit intrapulmonary artery (Altiere et al., 1994). However, recent findings suggest the contribution of muscarinic M_1 receptors to acetylcholine-induced relaxation in human pulmonary arteries and veins (Norel et al., 1996; Walch et al., 2000). These results suggest that other muscarinic receptor subtypes may also be involved in mediating acetylcholine vascular responses. In our study with conventional muscarinic receptor antagonists, only pirenzepine (a selective muscarinic M_1 receptor antagonist) and *p*-fluoro-hexa-hydro-sila-difenidol (*p*-FHHSiD) (a selective muscarinic M_3 receptor antagonist) managed to cause a parallel rightward shift of the acetylcholine concentration–response curves.

On the other hand, the observed competitive antagonism with pirenzepine and *p*-FHHSiD may suggest that both muscarinic M_1 and M_3 receptors are important in mediating the acetylcholine-induced pulmonary relaxation response. It is because the observed parallel rightward shift of the concentration–response curve by pirenzepine suggested a competitive antagonism. However, the estimated slope of Schild plot of pirenzepine was significantly different from unity, refuting the above suggestion. The ''apparent'' muscarinic M_1 antagonistic effect produced by pirenzepine may be explained by its limited receptor subtype selectivity (Caulfield, 1993). On the other hand, application of the selective muscarinic M_1 receptor agonist, 4-(3-chlorophenylcarbamoyloxy)2-butynyltrimethylammonium chloride (McN-A-343), failed to mimic the effects of acetylcholine in the pulmonary arterial preparations and produced no apparent relaxation response.

It is well known that not one of the conventional muscarinic receptor antagonists currently available is specific for any one muscarinic receptor subtype. They have nearly equal affinity for two to three of the muscarinic subtypes. For instance, the pA_2 value of pirenzepine obtained in the present study (7.1) was close to the apparent affinity value (6.9) for the interaction between pirenzepine and muscarinic M_3 receptors as determined either in functional (Eglen et al., 1994) or in radioligand binding (Dörje et al., 1991) studies. Moreover, the estimated pA_2 value 7.37 of *p*-FHHSiD was similar to its reported affinity on muscarinic M_1 (7.3) and M_3 (7.5) receptors (Eglen et al., 1996). Like pirenzepine and *p*-FHHSiD, other two muscarinic M_2 and M_4 receptor antagonists, methoctramine (the reported pA_2 for muscarinic M_2 receptor: 7.9) (Dörje et al., 1991) and tropicamide (the reported pA_2 for muscarinic M_4 receptor: 7.8) (Eglen et al., 1994), respectively, caused a rightward shift of the acetylcholine concentration–response curve. However, the estimated pA_2 values of these two agents (methoctramine, 6.44; tropicamide, 6.92) may argue the selectivity on the muscarinic M_2 and M_4 receptors, respectively. In conjunction with the slope of Schild plot that deviated from unity, the ''low'' pA_2 values of these two agents may reflect their low affinities on other muscarinic receptors such as the muscarinic M_3 receptor (pA_2: methoctramine, ~ 6.1; tropicamide, ~ 7.2) as reported previously (Dörje et al., 1991; Eglen et al., 1994). These results suggest that muscarinic M_2 and M_4 receptors might not be involved in acetylcholine-induced vasorelaxation. Nonetheless, it is interesting to note that the pA_2 value of various conventional muscarinic receptor antagonists estimated in our pharmacological study (using acetylcholine plus neostigmine) was ''fairly similar'' to the reported pA_2 value of individual receptor antagonists obtained from radioligand binding studies (Dörje et al., 1991).

On the other hand, the ''uncertainty'' regarding the subtype of muscarinic receptor involved was lessened by the observations that none of the highly selective muscarinic toxins (MT-3 and MT-7), from the Green Mamba *D. angusticeps*, now examined modified acetylcholine-induced relaxation. It has been documented that MT-3 has a K_i value of 2 nM for the muscarinic m4 receptor whereas MT-7 has a K_i value of 0.2 nM for the muscarinic m1 receptor. The highest concentration of these two toxins (30 nM) in this study should have had little/no effect on other muscarinic receptor subtypes (MT-3: K_i>78 nM for muscarinic m1 receptor and K_i>1 μM for muscarinic m2, m3 and m5 receptors; MT-7: K_i>2 μM for muscarinic m2, m3, m4 and m5 receptors) (reviewed by Bradley, 2000). These findings support the validity of our conclusion regarding the involvement of the muscarinic M_3 receptor in acetylcholine-elicited pulmonary artery relaxation. On the other hand, a possible role of the muscarinic m5 receptor in the generation and release of nitric

oxide, which contributes to acetylcholine-induced relaxation, had been suggested (Wang et al., 1994). Our pharmacological study, however, could not evaluate the possible participation of the muscarinic m5 receptor as there is no selective muscarinic m5 agonist or antagonist available.

As documented for other vascular preparations (Liu et al., 2001, Salom et al., 2001), removal of endothelium abolished the relaxation elicited by various neurotransmitters, suggesting that the relaxant is released from endothelium on activation of the endothelial muscarinic M_3 receptor. Our results showed that removal of endothelium abolished the relaxant responses, indicating that the vasorelaxing mediator(s) was also derived from the endothelium. To identify the mediator(s) released from the endothelium (Furchgott and Zawadzki, 1980; Gruetter et al., 1979; Palmer et al., 1987), the effect of a nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (Moncada et al., 1991), was studied. The failure of the arterial rings to exhibit relaxation in the presence of L-NAME demonstrated that nitric oxide synthesised from endothelial nitric oxide synthase was involved in vasorelaxation. Complete eradication of acetylcholine-mediated pulmonary arterial relaxation by L-NAME (20 μM) suggested that the vasodilatation observed was due solely to the nitric oxide released and that the putative endothelium hyperpolarizing factor, as observed in other vascular preparations (Coats et al., 2001; Karamsetty et al., 2001), may not participate in this response. Besides, a cyclo-oxygenase product such as prostacyclin, a potent vasodilator (Rang et al., 1999), is not important in mediating acetylcholine-induced pulmonary arterial vasodilatation as indomethacin (a nonselective cyclo-oxygenase inhibitor) did not alter the relaxation response. Our results were consistent with the observation that indomethacin produced no effect on acetylcholine-induced relaxation in rabbit aorta (Jaiswal et al., 1991). However, it has been reported that indomethacin enhances the relaxation induced by acetylcholine in rabbit intrapulmonary arteries (Altiere et al., 1994) whereas an inhibitory effect was recorded in human pulmonary arteries (Norel et al., 1996).

Activation of guanylate cyclase by nitric oxide to increase the guanosine 3,5-cyclic monophosphate (cGMP) level has been documented as a primarily pathway by which nitric oxide induces vasorelaxation (Hussain et al., 2001, Jiang and Dusting, 2001). Our results were also consistent with previous reports that preincubation with commonly used guanylate cyclase inhibitors, 6-anilino-5,8-quinolinedione (LY 83583) and methylene blue (Ignarro et al., 1984; Martin et al., 1985), markedly attenuated acetylcholine-mediated relaxation. We therefore concluded that acetylcholine-induced relaxation in the pulmonary arteries was achieved by activation of guanylate cyclase.

In addition to the well documented muscarinic M_3 receptor/IP_3/$[Ca^{2+}]_i$/eNOS intracellular second messenger pathway (Wylie et al., 1999) for the production of nitric oxide, recent evidence also suggests the participation of tyrosine kinase and phosphatidylinositol 3-kinase activation in acetylcholine-induced vasorelaxation of rat basilar artery (Kitazono et al., 1998; Kitayama et al., 2000) and both kinases are important for the generation of nitric oxide. In our study, neither genistein (a tyrosine kinase inhibitor) (Constantinou and Huberman, 1995) nor wortmannin (a phosphatidylinositol 3-kinase blocker) (Cross et al., 1995) was found to modify the relaxation response. Hence, in contrast to results with rat basilar artery, our results suggested that these two kinases were probably not involved in acetylcholine-induced pulmonary arterial relaxation.

$p21^{ras}$ is a member of a superfamily of related low molecular weight monomeric G proteins. $p21^{ras}$ and mitogen-activated protein kinases are involved in a variety of signal transduction pathways. Mitogen-activated protein kinases are divided into three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNKs) and p38 mitogen-activated protein kinase (Wylie et al., 1999). Extracellular signal-regulated kinase activation was shown to be $p21^{ras}$-dependent (Cano and Mahadevan, 1995) and can be activated by G-protein-coupled receptors. Recently, Wylie et al. (1999) reported that muscarinic m3 receptors expressed in Chinese hamster ovary cells were coupled to extracellular signal-regulated kinase activation. To explore the possible coupling of muscarinic m3 receptors to $p21^{ras}$ and extracellular signal-regulated kinases as well as to the NO-$p21^{ras}$-mitogen-activated protein kinases signalling pathway involved in acetylcholine-elicited pulmonary artery relaxation, experiments were performed in the presence of various modulators. Unfortunately, none of the modulators [(−)-perillic acid, a selective $p21^{ras}$ inhibitor (Schulz et al., 1997), 2-[2′-amino-3′-methoxy-phenyl]-oxanaphthalen-4-one (PD 98059, a p42/p44 mitogen-activated protein kinase inhibitor) (Lew et al., 1999) and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB 203580, a p38 mitogen-activated protein kinase inhibitor) (Young et al., 1997)] altered acetylcholine-induced pulmonary artery relaxation. These results may suggest that, in contrast to its role in other preparations, the $p21^{ras}$/mitogen-activated protein kinase pathway probably did not participate in mediating nitric oxide-elicited relaxation.

In conclusion, our results demonstrated that acetylcholine-induced pulmonary artery relaxation probably occurs through the activation of muscarinic M_3 receptor presence in the endothelium. The endothelium-dependent relaxation is mediated solely through the released nitric oxide whereas indomethacin-sensitive prostanoid(s) and endothelium dependent hyperpolarizing factor(s) are probably not involved. Moreover, the $p21^{ras}$/mitogen-activated protein kinase pathway did not contribute to acetylcholine-provoked pulmonary arterial relaxation.

4.1. Potential limitations

It is important to point out that we examined the *in vitro* relaxation response to acetylcholine in rat pulmonary artery and our results suggested that the endothelial muscarinic M_3

receptors are probably involved. However, there is evidence in the literature indicating that other subtype(s) of muscarinic receptors are involved in other species, including the human. Hence, caution is needed in extrapolation of the present observations to the human. Moreover, drugs that have been reported to inhibit effectively the $p21^{ras}$/mitogen-activated protein kinase pathways in other preparations failed to affect the acetylcholine-elicited nitric oxide-dependent pulmonary arterial relaxation we now observed. The ''apparent'' non-participation of the $p21^{ras}$/mitogen-activated protein kinase pathways should be interpreted carefully. The major problem was that only one concentration of these drugs was used and use of a higher concentration of these agents resulted in suppression of the phenylephrine-induced arterial tone.

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