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Indirect muscarinic receptor activation by pentamidine on airway smooth muscle

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1 Pentamidine is routinely used to reduce the incidence of *Pneumocystis carinii* pneumonia in patients infected with human immunodeficiency virus, but it has been described as inducing pulmonary adverse effects, such as cough and bronchospasm.

2 In this paper we have investigated the effects of pentamidine on guinea-pig isolated main bronchi and human isolated bronchi. Pentamidine induced a concentration-dependent contraction in both preparations with pD_2 values of 9.64 ± 0.07 ($n=8$) and 9.73 ± 0.06 ($n=8$) and a maximal effect (E_{max}) of $40 \pm 4\%$ and $34 \pm 5\%$ of the response to acetylcholine (1 mM) in guinea-pig and human bronchi respectively. Atropine (0.01 to 0.1 μM) and the muscarinic M_3 receptor antagonist, hexahydro-siladiphenidol (0.1 and 1 μM) inhibited pentamidine-induced concentration-responses in both preparations in a non-competitive manner, whereas only high concentrations of the M_1 receptor antagonist pirenzipine (1 μM) inhibited pentamidine concentration-response curves.

3 The cholinesterase inhibitor, tacrine (1 μM), potentiated the effect of pentamidine; in contrast, morphine inhibited pentamidine-induced responses.

4 The bronchoconstrictor effect of pentamidine on guinea-pig and human isolated bronchi was not modified by the H_1 histamine receptor antagonist, mepyramine, by indomethacin or by the neurokinin NK_1 and NK_2 receptor antagonists, CP-96,345 and SR 48969 respectively, suggesting that neither histamine receptor stimulation, arachidonic acid derivative formation, nor tachykinin release are involved in pentamidine-induced contraction of human and guinea-pig airways.

5 Our overall results suggest that pentamidine induces contraction of guinea-pig and human isolated bronchi through prejunctional cholinergic nerve stimulation.

Keywords: Pentamidine; muscarinic receptor; airway smooth muscle

Introduction

Aerosolized pentamidine is widely used for prevention and treatment of *Pneumocystis carinii* pneumonia in patients infected with human immunodeficiency virus (Masur, 1992). However, respiratory side-effects are frequently observed. Cough and bronchospasm have been reported to occur in up to 81% and 24% of patients respectively (TAPS, 1990; Montgomery *et al.*, 1995). The mechanisms of these side-effects remains so far unknown. In the guinea-pig *in vivo*, Jarreau *et al.* (1993) have suggested that tachykinins mediate both the bronchoconstrictor response and the increase in airway permeability induced by pentamidine in the guinea-pig *in vivo*. Tachykinins located in non-myelinated sensory nerve fibres of the airways and released after stimulation by various mechanical and chemical irritants may indeed induce cough and bronchospasm in human subjects (Fuller *et al.*, 1985). The involvement of tachykinin suggested by Jarreau *et al.* (1993) is, however, mainly based on the inhibitory effects of capsaicin and morphine, and the potentiating effects of the enkephalinase inhibitor, phosphoramidon, on pentamidine-induced bronchoconstriction and plasma extravasation. No study of the effects of specific NK_1 or NK_2 neurokinin receptor antagonists on pentamidine-induced bronchoconstriction have been performed to assess the involvement of tachykinins in their model.

The aim of this *in vitro* study to investigate the effect of pentamidine on guinea-pig main bronchi and on human isolated bronchi and, if a contractile effect was observed, to explore the mechanism of this action. To examine the tachykinin involvement suggested by Jarreau *et al.* (1993), we have studied the effects of tachykinin depletion by capsaicin and those of tachykinin NK_1 or NK_2 receptor antagonists, CP-96,345 and SR 48968 respectively, on pentamidine-induced contraction. Since pentamidine may have other actions, such as inhibition of cholinesterase (Altson, 1988), we have further investigated the potential contribution of a cholinergic reflex and of prostanoid or histamine release in pentamidine-induced smooth muscle contraction.

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Methods

Tissue preparation

Tricoloured guinea-pigs of either sex (250-350 g) anaesthetized with urethane (1.25 g kg^{-1}, i.p.) were killed by a blow on the head and the main bronchi removed and cut into rings.

Human bronchial tissues were obtained from patients (mean age, 60 years; range 42 to 76 years) undergoing surgery for lung cancer. All were male and previous smokers. None were asthmatic. Just after resection, segments of bronchi with an inner diameter of 1 to 3 mm were taken from an area as far as possible from the malignancy. They were placed in oxygenated Krebs-Henseleit solution (composition, mM: NaCl 119, KCl 5.4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.7) and stored overnight at 4°C. After removal of adhering fat and connective tissues, four to eight rings of the same bronchus were prepared.

Each set of human or guinea-pig airway rings was suspended under an initial tension of 2 g in Krebs-Henseleit solution, bubbled with 95% O_2:5% CO_2 and maintained at 37°C. Changes in force of contraction were measured isometrically with strain gauge amplifiers and displayed on I.O.S.-Moise 3 recorder (EMKA, Mitry Mory, France). At the beginning of each experiment, the bronchial strips were con-

tracted with acetylcholine (ACh 1 mM), and then maximal relaxation was induced by theophylline (3 mM). During the next 90 min, the tissues were washed every 15 min. Experiments were conducted on parallel groups of 4 to 8 rings, one ring serving as control.

Protocol

Concentration-response curves to pentamidine (0.1 nM to 10 nM) or acetylcholine (10 nM to 1 mM) were recorded by applying increasing concentrations of drugs every 3 to 6 min and 5 to 10 min respectively in logarithmic increments. Contractile responses were expressed as a percentage of the effect induced by ACh (1 mM).

Pretreatments with capsaicin (10 μM), the NK_2 receptor antagonist, SR 48968 (1 μM), the NK_1 receptor antagonist, CP-96,345 (1 μM), cromoglycate (1 μM), atropine (1 nM to 1 μM), pirenzepine (0.1 μM to 10 μM) or mepyramine (1 μM) were made 1 hour before cumulative concentration-response curves (CRC) to pentamidine or acetylcholine. Pretreatments with phosphoramidon (1 μM), tetrodotoxin (1 μM), indomethacin (1 μM), hexahydro-sila-diphenidol (10 nM to 1 μM), morphine (1 μM), or tacrine (1 μM) were made 15 min before cumulative concentration-response curves to pentamidine.

Responses were expressed as percentages of contraction induced by ACh (1 mM).

Statistical analysis

The potencies of agonists were defined as pD_2, i.e. the negative log of the drug concentration that caused 50% of the maximal effect induced by the drug ($pD_2 = -\log EC_{50}$). Maximal effects (E_{max}) were expressed as percentage of contraction induced by ACh (1 mM). Statistical analysis of the results was performed by variance analysis and Student's t test. All values in the text and in the figures are expressed as mean ± standard error of the mean (s.e.mean). P values lower than 0.05 were considered to be significant.

Drugs

The substances used were: acetylcholine, morphine hydrochloride (PCH, Paris, France); SR 48968: (S)-N-methyl-N[4-(4-acetyl-amino-4-phenylpiperidino)-2-(3,4-dichlorophenyl-butyl]benzamide, CP-96,345: (-)-*cis*-2-(diphenylmethyl)-N-[2-methoxyphenyl)-methyl]-1-azabicyclo-[2.2.2]-octan-3-amine (Sanofi-Recherche, Montpellier, France); indomethacin, atropine sulphate, phosphoramidon, pirenzepine dihydrochloride, mepyramine, capsaicin, cromoglycate, tetrodotoxin (Sigma, St Louis, U.S.A.); theophylline sodium anisate (Bruneau, Paris, France); pentamidine isethionate (Pentacarinat; Roger Bellon, Neuilly-sur-seine, France); hexahydro-sila-diphenidol, tacrine (RBI, Bioblock, Illkirch, France). All drugs were dissolved in distilled water and then diluted in Krebs solution, except for indomethacin, SR 48968 and CP-96,345, which were dissolved in ethanol and then diluted in Krebs solution. The maximal amount of ethanol added to the bath (0.4%) did not alter reactivity of preparations to acetylcholine.

Results

Effect of pentamidine on guinea-pig isolated main bronchi and human isolated bronchi

Pentamidine (0.1 nM to 10 nM) induced a concentration-dependent contraction of both guinea-pig isolated main bronchi and human isolated bronchi, with pD_2 values of 9.64 ± 0.07 ($n = 8$) and 9.73 ± 0.06 ($n = 8$) and an E_{max} of 40 ± 4% and 34 ± 5% respectively. The potency and efficacy of pentamidine were similar in both preparations (Figure 1).

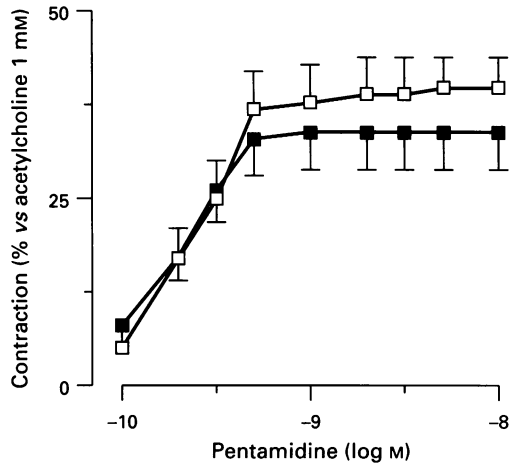

Figure 1 Effect of pentamidine (0.1 nM to 10 nM) on the guinea-pig isolated main bronchus (□) and the human isolated bronchus (■). Values are means ± s.e.mean ($n = 8$).

Role of tachykinins in pentamidine-induced contractions of guinea-pig main bronchi

In vitro C fibre desensitization by 1 h capsaicin (10 μM) pretreatment (Maggi *et al.*, 1990; Mapp *et al.*, 1991), inhibition of nerve conduction by the sodium channel blocker, tetrodotoxin (1 μM), and C fibre 'stabilization' by cromoglycate (1 μM) (Dixon *et al.*, 1980) did not significantly inhibit pentamidine-induced guinea-pig isolated main bronchi contraction ($n = 6$, data not shown). The enkephalinase inhibitor, phosphoramidon (1 μM) did not significantly potentiate pentamidine-induced contraction of guinea-pig main bronchi ($n = 6$, data not shown). Finally, neither the NK_1 tachykinin receptor antagonist, CP-96,345 (1 μM), nor the NK_2 tachykinin receptor antagonist, SR 48968 (1 μM) either alone or combined, significantly inhibited the pentamidine-induced contraction of guinea-pig main bronchi ($n = 6$, data not shown).

Role of prostanoids and histamine in pentamidine-induced contractions

The effects of pentamidine on guinea-pig isolated main bronchi were not modified by the cyclo-oxygenase inhibitor indomethacin (1 μM) or by the histamine H_1 receptor antagonist, mepyramine (1 μM) ($n = 6$, data not shown).

Effect of pentamidine on acetylcholine-induced contraction

Pentamidine (0.1 nM to 1 nM) did not modify the acetylcholine (10 nM to 0.3 mM)-induced contractions of guinea-pig main bronchi ($pD_2 = 6.2 \pm 0.3$ and 6 ± 0.4 in control and after pentamidine 1 nM pretreatment respectively, $n = 5$).

Role of muscarinic receptor activation in pentamidine-induced contraction of guinea-pig main bronchi

Atropine (0.001 to 0.1 μM) inhibited or abolished the contractile effect of pentamidine in guinea-pig main bronchi ($n = 4$) (Figure 2a). The muscarinic M_1 receptor antagonist, pirenzepine, inhibited pentamidine-induced contraction of guinea-pig main bronchi only at concentrations higher than (0.1 μM) ($n = 5$) (Figure 2b). Finally, the effects of pentamidine on guinea-pig main bronchi were inhibited by the muscarinic M_3 receptor antagonist, hexahydro-sila-diphenidol (0.1 and 1 μM) ($n = 6$) (Figure 2c).

Mechanism of contractile effect of pentamidine in human isolated bronchi

The effects of pentamidine in human isolated bronchi were not inhibited by the NK_1 receptor antagonist, CP-96,345 (1 μM), the NK_2 receptor antagonist, SR 48968 (1 μM) alone or combined, or by indomethacin (1 μM) or mepyramine (1 μM) ($n=6$, data not shown) but were inhibited by atropine (1 μM) ($n=6$) (Figure 3a) and by the muscarinic M_3 receptor antagonist, hexahydro-sila-diphenidol (0.1 and 1 μM) ($n=6$) (Figure 3b).

Effect of cholinesterase inhibition by tacrine on pentamidine and acetylcholine-induced contraction of guinea-pig and human isolated bronchi

Figure 4 shows that after 15 min incubation of human isolated bronchi with tacrine (1 μM) ($n=6$), the concentration-response curves to pentamidine (4a) or to acetylcholine (4c) were significantly shifted to the left and the maximal effect of pentamidine was increased. Similar results were observed on guinea-pig main bronchi (Figure 4b, 4d).

Effect of morphine on the pentamidine-induced contraction of guinea-pig and human isolated bronchi

Morphine (1 μM) inhibited the contractile effect of pentamidine in guinea-pig main bronchi ($n=6$) (Figure 5a) and in human isolated bronchi ($n=6$) (Figure 5b). Under similar conditions, morphine 1 μM, had no effect on acetylcholine-

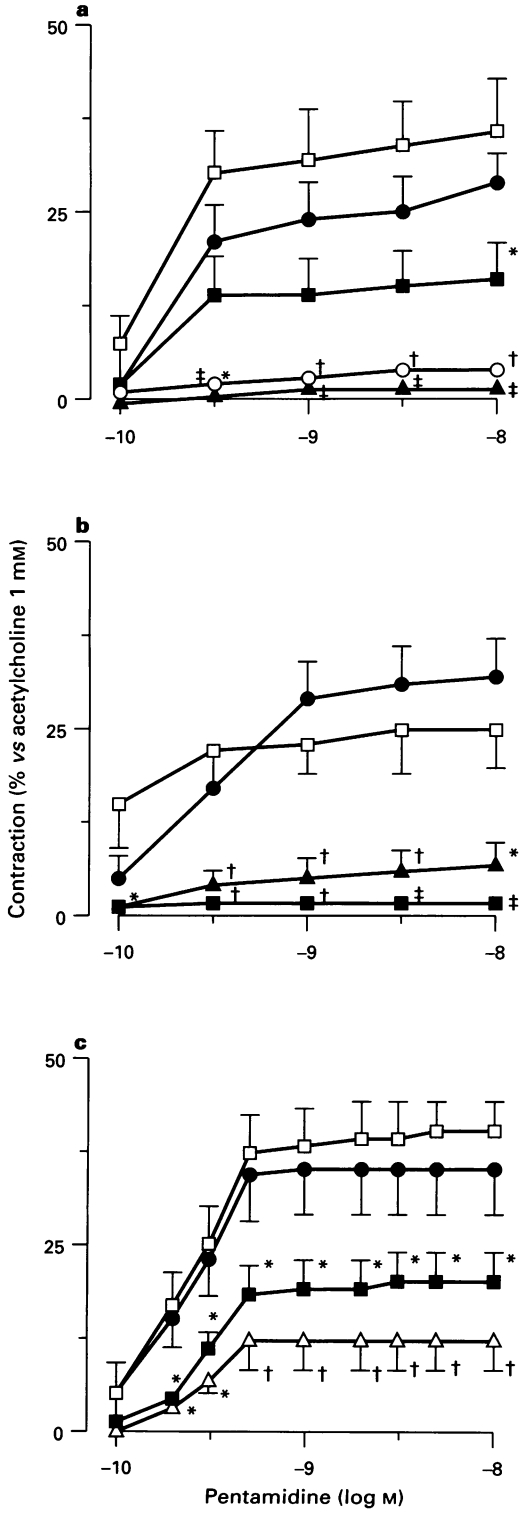

Figure 2 Effect of (a) atropine ($n=4$), (b) the M_1 receptor antagonist, pirenzepine ($n=5$), (c) the M_3 receptor antagonist, hexahydro-sila-diphenidol (HHSiD) ($n=6$) on pentamidine-induced contraction of guinea-pig isolated main bronchi. Control (□), atropine (1 nM ●; 10 nM ■; 30 nM ○; 0.1 μM; ▲), pirenzepine (0.1 μM ●; 1 μM ▲; 10 μM ■), hexahydro-sila-diphenidol (10 nM ●; 0.1 μM ■; 1 μM △). Results are shown as means ± s.e.mean. Significant differences from control are shown as: $*P<0.05$; $\dagger P<0.01$; $\ddagger P<0.001$.

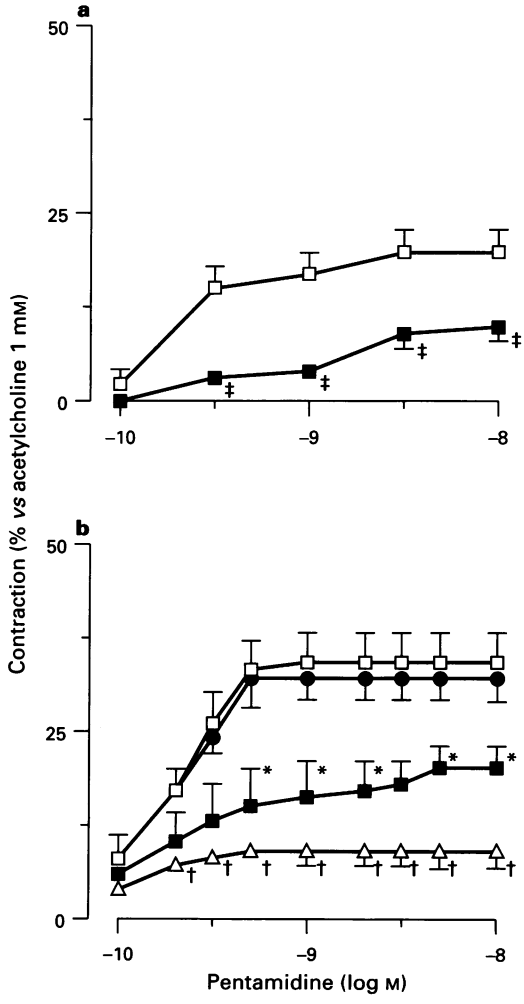

Figure 3 Effect of (a) atropine ($n=6$), (b) the M_3 receptor antagonist, hexahydro-sila-diphenidol (HHSiD) ($n=6$) on pentamidine-induced contraction of human isolated bronchi. Control (□), atropine (1 μM ■), hexahydro-sila-diphenidol (10 nM ●; 0.1 μM ■; 1 μM △). Results are shown as means ± s.e.mean. Significant differences from control are shown as: $*P<0.05$; $\dagger P<0.01$; $\ddagger P<0.001$.

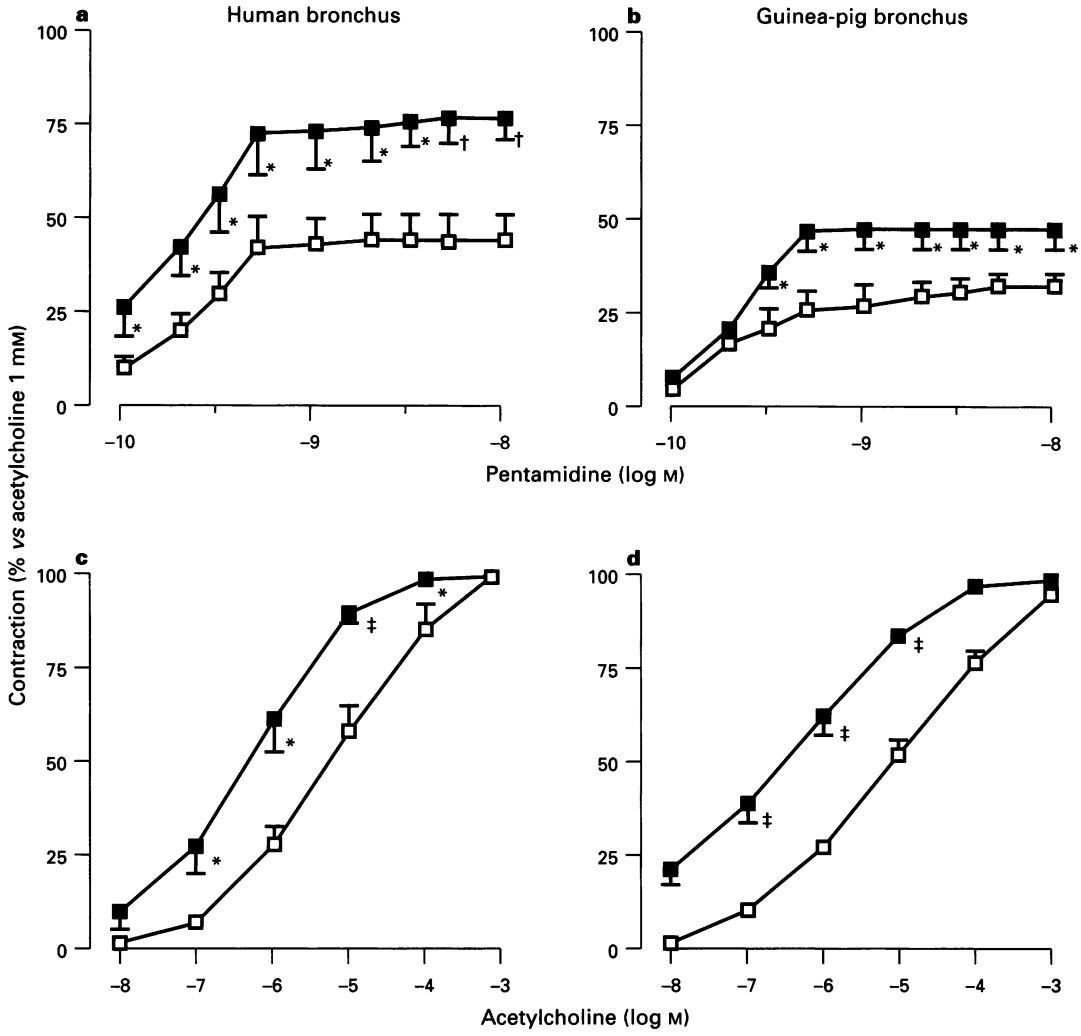

Figure 4 Effect of tacrine ($n=6$) on pentamidine (0.1 nM to 10 nM) (a,b) and acetylcholine (10 nM to 1 mM) (c,d)-induced contraction of human isolated bronchus (a,c) and guinea-pig isolated main bronchus (b,d). Control (□), tacrine (1 μM ■). Results are shown as means ± s.e.mean. Significant differences from control are shown as $*P<0.05$; $\dagger P<0.01$; $\ddagger P<0.001$.

induced contraction ($pD_2 = 5.2 \pm 0.2$ and $pD_2 = 5.1 \pm 0.4$ in the absence and the presence of morphine respectively in human bronchi, $n=6$).

Discussion

Our results demonstrate that pentamidine is a potent but moderately efficacious contractile agent on guinea-pig and human isolated bronchi. Aerosolized pentamidine is known to induce cough (TAPS, 1990), suggesting that neural reflex could be involved in its effects. Recently, in an *in vivo* study, Jarreau *et al.* (1993) inhibited with capsaicin and morphine pentamidine-induced airway constriction and microvascular leakage in guinea-pig *in vivo*, and suggested that tachykinins could be involved in the respiratory side effects of pentamidine. Several pieces of evidence suggest that the capsaicin effect may not be entirely via inhibition of tachykinin release in human bronchi. Indeed, we have demonstrated previously that capsaicin inhibits bradykinin-induced airway smooth muscle contraction whereas bradykinin-induced contraction is not inhibited by potent and specific NK_1 and NK_2 receptor antagonists (Molimard *et al.*, 1994). Furthermore, interaction of capsaicin with prostanoid synthesis has been reported (Juan *et al.*, 1980; Flynn *et al.*, 1986). The involvement of tachykinin would have been definitively assessed in the model of Jarreau *et al.* (1993) by showing an inhibitory effect of specific NK_1 or NK_2 receptor antagonists. We also studied the role of C fibres in pentamidine-induced isolated bronchi contraction. We demonstrated that the Na^+ channel blocker, tetrodotoxin failed to inhibit pentamidine-induced contraction of guinea-pig isolated main bronchi, thereby showing that nerve conduction is not involved in this contraction. Capsaicin pretreatment, C fibre stabilization by sodium cromoglycate or neurokinin NK_1 and NK_2 receptor blockade by high concentration of CP-96,345 (1 μM) and SR 48968 (1 μM) also failed to inhibit pentamidine-induced contraction of either guinea-pig or human airways, suggesting that tachykinins are not involved in pentamidine-induced isolated airway contraction, conversely to what is suggested in guinea-pig *in vivo*.

Our results are at variance with those of Jarreau *et al.* (1993) and these differences could be due to the relative importance of microvascular leakage and smooth muscle contraction in airways resistance. Indeed, it seems likely that the aerosolized pentamidine-induced increase of respiratory system resistance observed *in vivo* is mainly caused by airway oedema, as confirmed by the lack of reversibility of the long-lasting increase in respiratory resistance after hyperinflation and the histological findings shown by Jarreau *et al.* (1993). Thus, airway smooth muscle contraction could play only a modest role in the increase in airway resistance seen after aerosolized pentamidine, as suggested by the low efficiency of pentamidine on isolated airways. The use of specific neurokinin NK_1 and NK_2 receptor

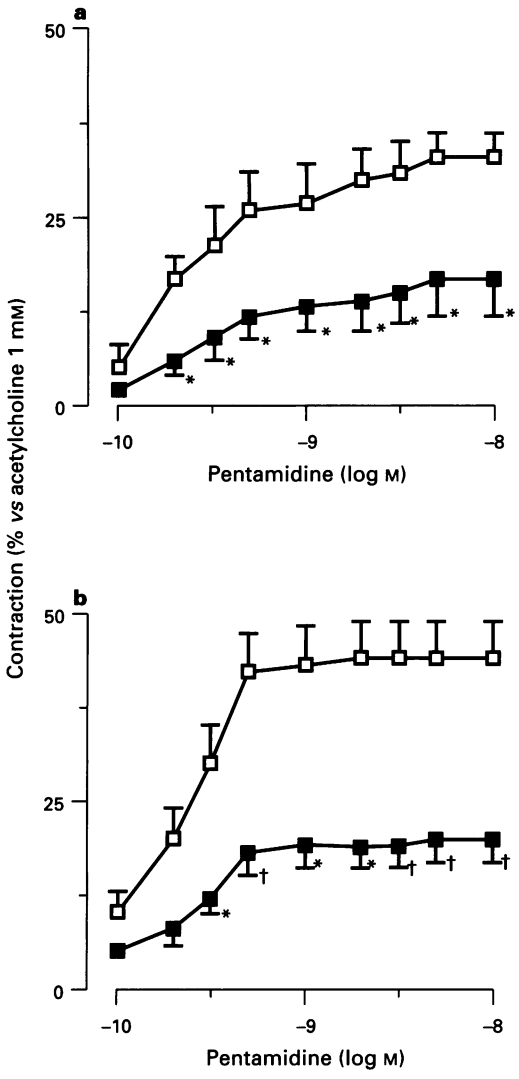

Figure 5 Effect of morphine ($n=6$) on pentamidine-induced contraction of guinea-pig isolated main bronchus (a) and human isolated bronchus (b). Control (□), morphine (1 μM ■). Results are shown as means ± s.e.mean. Significant differences from control are shown as: $*P<0.05$; $\ddagger P<0.01$.

antagonists in the Jarreau *et al.* (1993) study would have clarified the relative importance of microvascular leakage and smooth muscle contraction.

We then studied further the potential involvement of other contractile mechanisms of action of pentamidine, such as cholinoceptor stimulation, histamine or prostanoid release. Indeed, pentamidine may have other actions, such as inhibition of cholinesterase (Altson 1988). Furthermore, the sulphonate salt of pentamidine isethionate contains sulphite (Debs *et al.*, 1990) which could have a bronchoconstrictor effect not mediated by tachykinin or by acetylcholine release (Lötvall *et al.*, 1990). Histamine or contractile prostanoid release by pentamidine can be ruled out, since the histamine H_1 antagonist, mepyramine and indomethacin failed to inhibit pentamidine-induced contraction.

Our results show that pentamidine-induced contraction is inhibited by atropine, suggesting that a cholinergic mechanism is involved in pentamidine-induced human and guinea-pig isolated airways contraction. A cholinergic reflex is unlikely to be involved since tetrodotoxin did not inhibit pentamidine-induced contraction. Additionally, pentamidine-induced cholinergic reflex activation by muscarinic M_1 receptor stimulation may also be ruled out since pirenzepine at a concentration known to be specific for the muscarinic M_1 receptor (0.1 μM) (Levine & Birdsall, 1989) failed to inhibit pentamidine-induced contraction. At higher concentrations pirenzepine may antagonize M_2 and M_3 receptors (Choo & Mitchelson, 1985; Levine & Birdsall, 1989) and also inhibit pentamidine-induced contraction. The muscarinic M_3 receptor has been described as mediating acetylcholine-induced contraction of smooth muscle (Eglen *et al.*, 1994). We have therefore studied the effect of the M_3 receptor antagonist hexahydro-sila-diphenidol hydrochloride. The effect of pentamidine on guinea-pig and human isolated bronchi were inhibited by the M_3 receptor antagonist, showing that pentamidine-induced contractions are via M_3 muscarinic receptors.

However, we were surprised by the non-competitive antagonistic effect of atropine on pentamidine-induced contractions, as atropine is a well-known competitive antagonist of acetylcholine on M_3 receptors. These results suggest that the M_3 receptor stimulation by pentamidine could be indirect (Kenakin, 1993). We therefore studied the effect of morphine which has been demonstrated to inhibit acetylcholine release evoked by electrical field stimulation in guinea-pig main bronchi (Belvisi *et al.*, 1990). Our results show that, although the effects of acetylcholine were unmodified by morphine, pentamidine-induced contractions were inhibited by morphine, suggesting that the indirect contractile effect of pentamidine via M_3 receptors could be due to a release of acetylcholine from cholinergic nerve endings. This was further supported by the potentiating effect of cholinesterase inhibitor, tacrine, on pentamidine-induced contraction. Indeed, we have shown that as expected from cholinesterase inhibitor, tacrine, caused a shift of the concentration-response curve to acetylcholine without affecting the acetylcholine maximal effect. Tacrine increases the maximal effect of pentamidine instead of inducing a shift of the concentration-response curve, in agreement with an indirect effect of pentamidine. Indeed, the chemical structure devoid of ester function of pentamidine makes it unlikely that pentamidine is sensitive to cholinesterase.

A cholinesterase inhibitory effect of pentamidine (0.1 mM), suggested from earlier work with human plasma (Altson *et al.*, 1988), that could potentiate acetylcholine effects has also been ruled out by the lack of the potentiating effect of pentamidine on acetylcholine-induced contraction.

In conclusion, we have demonstrated that pentamidine is a potent guinea-pig and human bronchi contractile agent, and we suggest that this effect is due to a prejunctional stimulation of cholinergic nerves inducing the release of acetylcholine. The use of cholinoceptor antagonists (atropine or ipratropium bromide) in human subjects could clarify the respective roles of microvascular leakage and smooth muscle contraction in aerosolized pentamidine-induced bronchospasm.

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