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Effect of salmeterol on human nasal epithelial cell ciliary beating: inhibition of the ciliotoxin, pyocyanin

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1 Patients with airway infection by *Pseudomonas aeruginosa* have impaired mucociliary clearance. Pyocyanin is a phenazine pigment produced by *P. aeruginosa* which is present in the sputum of colonized patients, slows human ciliary beat frequency (CBF) *in vitro* and slows mucociliary transport *in vivo* in the guinea-pig.

2 We have investigated the effect of salmeterol, a long-acting β_2-adrenoceptor agonist, on pyocyanin-induced slowing of human CBF *in vitro*. Salmeterol (2×10^{-7} M) was found to reduce pyocycanin ($20\ \mu g\ ml^{-1}$)-induced slowing of CBF by 53% and the fall in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) by 26% and ATP by 29%.

3 Another β_2-adrenoceptor agonist, isoprenaline (2×10^{-7} M), also inhibited pyocyanin-induced slowing of CBF by 39%.

4 The effects of salmeterol (30 min preincubation) persisted after washing the cells.

5 Propranolol (10^{-7} M) and the β_2-specific antagonist, ICI 118551 (10^{-6} M) blocked the protective effects of salmeterol completely, but atenolol (10^{-6} M) was less effective. These results suggested that the effects of salmeterol on pyocyanin-induced effects were mediated primarily via the stimulation of β_2-adrenoceptors.

6 Pyocyanin-induced ciliary slowing is associated with a substantial fall in intracellular cyclic AMP and ATP. Salmeterol reversed the effects of pyocyanin on cyclic AMP and ATP.

7 Mucociliary clearance is an important defence mechanism of the airways against bacterial infection. Salmeterol may benefit patients colonized by *P. aeruginosa*, not only by its bronchodilator action, but also by protecting epithelial cells from pyocyanin-induced slowing of CBF.

Keywords: Salmeterol; cilia; ciliotoxin; pyocyanin; *Pseudomonas aeruginosa*; β agonist; cyclic AMP; ATP

Introduction

Many patients with chronic airways infection by bacteria such as *Pseudomonas aeruginosa* have airflow obstruction and impaired mucociliary clearance (Hoiby, 1982; Fick, 1989; Cole & Wilson, 1989). Pyocyanin is a phenazine pigment produced by *P. aeruginosa* which is present in the sputum of colonized patients at high concentrations up to $27\ \mu g\ ml^{-1}$ (Wilson *et al.*, 1988). Pyocyanin slows human ciliary beat frequency (CBF) *in vitro*, and later disrupts the integrity of the epithelial surface (Wilson *et al.*, 1987); it also slows tracheal mucociliary transport in guinea-pigs *in vivo* (Munro *et al.*, 1989). *P. aeruginosa* also produces 1-hydroxyphenazine (Wilson *et al.*, 1987) and rhamnolipid (Read *et al.*, 1992) which both slow human ciliary beating, and collectively these products are likely to play a part in bacterial colonisation of the lower respiratory tract. We have recently shown that pyocyanin-induced slowing of human ciliary beat *in vitro* is associated with a fall in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) and adenosine 5'-triphosphate (ATP). Ciliary beat slowing could be prevented by treating the epithelial cells with agents known to increase cyclic AMP levels (Kanthakumar *et al.*, 1993).

Studies investigating the effects of inhaled β_2-adrenoceptor agonists on mucociliary clearance in humans *in vivo* have indicated that this may be an important aspect of their therapeutic efficacy, especially in infective lung disease (Pavia, 1984; Devalia *et al.*, 1992). The mechanism is unclear but it has been suggested that it involves increase of ciliary activity and intracellular cyclic AMP levels by stimulation of β_2-adrenoceptors on the epithelial surface (Devalia *et al.*, 1992; Lansley *et al.*, 1992). Studies by Verdugo and colleagues (1980) and Di Benedetto and colleagues (1991) have suggested that cyclic AMP is a regulator of ciliary activity in airway epithelium, possibly by affecting the availability or use of ATP by the ciliary axoneme (Lansley *et al.*, 1992). Salmeterol is a potent β_2 agonist with a prolonged action and causes a greater increase in intracellular cyclic AMP than salbutamol (Devalia *et al.*, 1992). We have investigated the effect of salmeterol on pyocyanin-induced changes in ciliary beating, and intracellular cyclic AMP and ATP levels, in human nasal epithelial cells, and have compared salmeterol with isoprenaline.

Methods

Human nasal epithelium

Strips of normal human nasal ciliated epithelium were obtained with a cytology brush (Rutland & Cole, 1980) from the inferior turbinate of healthy volunteers who had been free of respiratory infection for at least 4 weeks and dispersed by agitation in cell culture medium 199 with Earle's salts and HEPES (N-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) (Flow Laboratories, Canada). The sample of epithelium was divided, and sealed microscope coverslip-slide preparations were constructed for assessment by light microscopy. CBF was measured photometrically from ten identified sites on at

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least six strips of epithelium at 37°C as previously described (Wilson et al., 1987; 1988; Kanthakumar et al., 1993). Ciliary dyskinesia (loss of the normal coordinated pattern of ciliary beating), ciliostasis (absence of ciliary beating), and disruption of the integrity of the epithelial surface (irregularity and break-up of the previously smooth intact surface) were recorded when present. Although every effort was made to make recordings of CBF at the same point of a strip at each experimental time-point, this was not always possible because epithelial strips sometimes move or change shape during an experiment because of ciliary beating. For this reason a recording of zero CBF was made only when ciliary beating had ceased on the whole epithelial strip. A sample of epithelium from a volunteer was used for only one experiment. Each experiment was repeated on six separate occasions with nasal epithelial samples obtained from six separate volunteers.

Effect of salmeterol on pyocyanin-induced ciliary slowing

Pyocyanin ($20\ \mu g\ ml^{-1}$) was used in all experiments. This concentration has been observed in the sputum of infected patients (Wilson et al., 1988), and in previous experiments in our laboratory (Kanthakumar et al., 1993) caused CBF to fall to about half of control by 4 h, disruption of epithelium integrity at 3 h, and marked falls in both intracellular cyclic AMP and ATP. For each experiment the sample of nasal epithelial strips in medium 199 was divided into four equal aliquots. Four sealed microscope coverslip-slide preparations were then constructed containing medium 199 alone, pyocyanin ($20\ \mu g\ ml^{-1}$), salmeterol ($1-4 \times 10^{-7}$ M) or salmeterol and pyocyanin. Epithelium for preparations containing salmeterol was preincubated with salmeterol for 15 min. The preparations were allowed to stabilize for a further 15 min after slide construction, and then CBF was measured at hourly intervals for 4 h. The experiment was repeated with isoprenaline (2×10^{-7} M).

One aliquot of nasal epithelial strips was pre-incubated with salmeterol (2×10^{-7} M) for 5 min, 15 min or 30 min. The aliquots were washed twice in medium 199. Each aliquot was centrifuged (800 g, 5 min) to pellet the epithelium, the supernatant was carefully removed with a pipette, and the epithelium resuspended in fresh medium 199. This procedure was repeated a second time. Four microscope-slide preparations were then constructed containing medium 199 alone, pyocyanin (epithelium not exposed to salmeterol), pyocyanin (epithelium incubated with salmeterol for 5, 15 or 30 min), and pyocyanin and salmeterol (present throughout the experiment, as in the concentration-response experiments described above).

Effect of salmeterol on pyocyanin-induced ciliary slowing in the presence of β-adrenoceptor antagonists

For each series of experiments, samples of nasal epithelium were incubated with salmeterol (2×10^{-7} M, 30 min at 37°C), washed twice and then exposed to pyocyanin ($20\ \mu g\ ml^{-1}$) and either propranolol (10^{-7} M), ICI 118551 (10^{-6} M) or atenolol (10^{-6} M). Five microscope-slide preparations were constructed for each experiment containing medium 199 alone, β-adrenoceptor antagonist, pyocyanin, pyocyanin (epithelium incubated with salmeterol), and pyocyanin and β-antagonist (epithelium incubated with salmeterol). Atenolol is a selective β_1-antagonist ($pA_2\ \beta_1 = 7.1$, $pA_2\ \beta_2 = 5.6$; O'Donnell & Wanstall, 1979) and ICI 118551 a selective β_2 antagonist ($pA_2\ \beta_2 = 8.8$, $pA_2\ \beta_1 = 6.7$; O'Donnell & Wanstall, 1981). Therefore at the concentrations of atenolol and ICI 118551 used, selective blockade of β_1 and β_2 adrenoceptors respectively was achieved.

ATP and cyclic AMP measurement

Levels of ATP in nasal epithelium were measured spectrophotometrically (Sigma, UK). Cyclic AMP levels were measured by enzyme immunoassay (Amersham, UK) (Kanthakumar et al., 1993). Nasal epithelial samples ($n = 6$) were treated with pyocyanin ($20\ \mu g\ ml^{-1}$) for 2 h in the presence or absence of salmeterol (2×10^{-7} M). This incubation time was chosen since the effects of pyocyanin are completely reversible at this point (Kanthakumar et al., 1993). Cellular protein levels were assessed by the method of Lowry et al. (1951), and levels of adenosine nucleotides were expressed as mg^{-1} of cellular protein.

Materials

Pyocyanin was prepared by photolysis of phenazine methosulphate (Knight et al., 1979) (Aldrich Chemicals, U.S.A.) and purified and characterized by its h.p.l.c.-u.v. profile as previously described (Wilson et al., 1988). Pyocyanin was tested at a final concentration of $20\ \mu g\ ml^{-1}$ ($\sim 10^{-4}$ M) in all experiments. Salmeterol hydroxynaphthoate (Glaxo, UK) was initially dissolved in glacial acetic acid and then diluted in phosphate buffered saline (PBS, pH 7.0); isoprenaline sulphate (Sigma, UK) was dissolved in distilled water. The β_2-specific antagonist, erythro-DL-1(7-methylindan-4-yloxy)-3-iso propylamino-butan-2-ol (ICI 118551, ICI, UK; Lemoine et al., 1988) was dissolved in PBS (pH 7.0). The β_1-specific antagonist, atenolol (Sigma, UK) and the non-specific β-antagonist, propranolol hydrochloride (Sigma, UK) were dissolved in distilled water.

Statistical analysis

Statistical analysis was carried out with the Wilcoxon signed ranks paired test. Data were expressed as mean ± standard error of the mean (s.e.mean). The mean percentage inhibition of pyocyanin-induced effects (protection) afforded by salmeterol and isoprenaline was calculated after 4 h as 100 × [mean ($n = 6$) CBF of pyocyanin with β-agonist − mean ($n = 6$) CBF of pyocyanin without β agonist]/[mean ($n = 6$) CBF of control − mean ($n = 6$) CBF of pyocyanin without β-agonist].

Results

Effect of salmeterol on pyocyanin-induced ciliary slowing

Salmeterol (1, 2 and 4×10^{-7} M) had no effect on baseline CBF compared to control CBF in medium 199 alone. Pyocyanin caused progressive slowing of CBF over 4 h (58% compared with control cells). Epithelial disruption was observed at 3 h and ciliary dyskinesia at 4 h. All three concentrations of salmeterol reduced pyocyanin-induced ciliary slowing with mean percentage protection at 4 h of 37%, 53% and 60% for 1, 2 and 4×10^{-7} M, respectively (Figure 1). Ciliary dyskinesia was not observed in any of the experiments incorporating salmeterol. Salmeterol (2 and 4×10^{-7} M) delayed the appearance of epithelial disruption from 3 h to 4 h. A salmeterol concentration of 2×10^{-7} M was chosen for use in all further experiments.

In a separate series of experiments, the effect of washing the tissue after exposure to salmeterol, before incubation with pyocyanin was studied. Incubation of epithelial cells with salmeterol (2×10^{-7} M) for 5 or 15 min, before washing, had no significant effect on pyocyanin-induced ciliary slowing, ciliary dyskinesia or epithelial damage. However, incubation with salmeterol for 30 min before washing produced a similar effect to that produced with salmeterol present throughout the experiment (Figure 2).

Effect of salmeterol on pyocyanin-induced ciliary slowing in the presence of β-adrenoceptor antagonists

Propranolol (10^{-7} M; Figure 3a) and ICI 118551 (10^{-6} M, Figure 3b) completely abolished the protective effect afforded

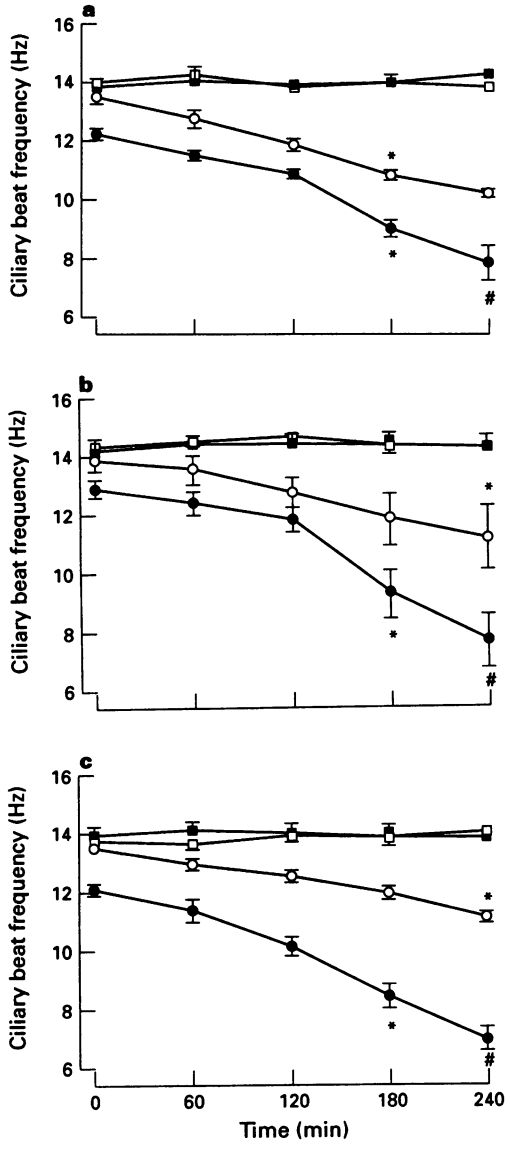

Figure 1 The effect of salmeterol on pyocyanin-induced ciliary beat slowing. Salmeterol was present throughout the experiment. Salmeterol alone (□) had no effect on baseline ciliary beat frequency (CBF) compared to control CBF in medium 199 alone (■) at any of the concentrations tested: (a) 1×10^{-7} M, (b) 2×10^{-7} M or (c) 4×10^{-7} M. Salmeterol (○) at all three concentrations significantly ($P<0.05$) reduced ciliary slowing and ciliary dyskinesia (#) produced by pyocyanin (●). The two higher concentrations were also able to delay the appearance of epithelial disruption (*) by 1 h.

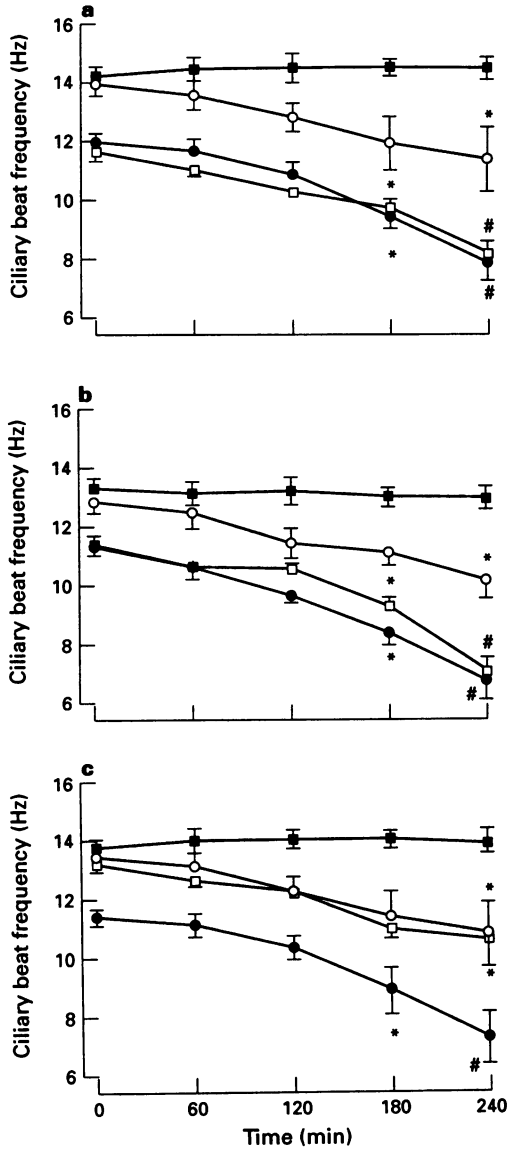

Figure 2 The effect of preincubation time on the protection afforded by salmeterol on pyocyanin-induced ciliary beat slowing. Incubation of cells with salmeterol (2×10^{-7} M) (□) for (a) 5 min and (b) 15 min followed by washing did not influence pyocyanin-induced ciliary beat slowing (●), but (c) incubation for 30 min gave equivalent results to salmeterol present throughout the experiment (○) (e.g. Figure 1b). Control medium 199 alone (■), # = ciliary dyskinesia, * = epithelial disruption.

by salmeterol (2×10^{-7} M) on pyocyanin-induced ciliary slowing, ciliary dyskinesia and epithelial disruption. Atenolol (10^{-6} M) partially inhibited the protective effect of salmeterol (2×10^{-7} M) on pyocyanin-induced ciliary slowing (Figure 3c), but had no effect on the protection afforded by salmeterol against pyocyanin-induced ciliary dyskinesia and epithelial disruption.

Effect of isoprenaline on pyocyanin-induced ciliary slowing

Isoprenaline (2×10^{-7} M) alone had no effect on baseline CBF, but significantly protected (39% inhibition) against pyocyanin-induced slowing of CBF; this effect compared with 53% inhibition by salmeterol at the same concentration. Isoprenaline also prevented ciliary dyskinesia, and delayed the appearance of epithelial disruption by 1 h.

Effect of salmeterol on pyocyanin-induced fall in intracellular cyclic AMP and ATP

Incubation of epithelial cells with pyocyanin for 2 h produced a 93% fall in intracellular cyclic AMP and 88% fall in ATP levels compared with controls (Figure 4). Incubation of cells with salmeterol (2×10^{-7} M) had no effect on basal levels of either nucleotide but did significantly protect against pyocyanin-induced falls in both cyclic AMP (26% protection), and ATP (29% protection).

Discussion

Pyocyanin is a phenazine pigment produced by *P. aeruginosa* that has a number of important effects during infections of the respiratory tract. Pyocyanin slows ciliary beating and

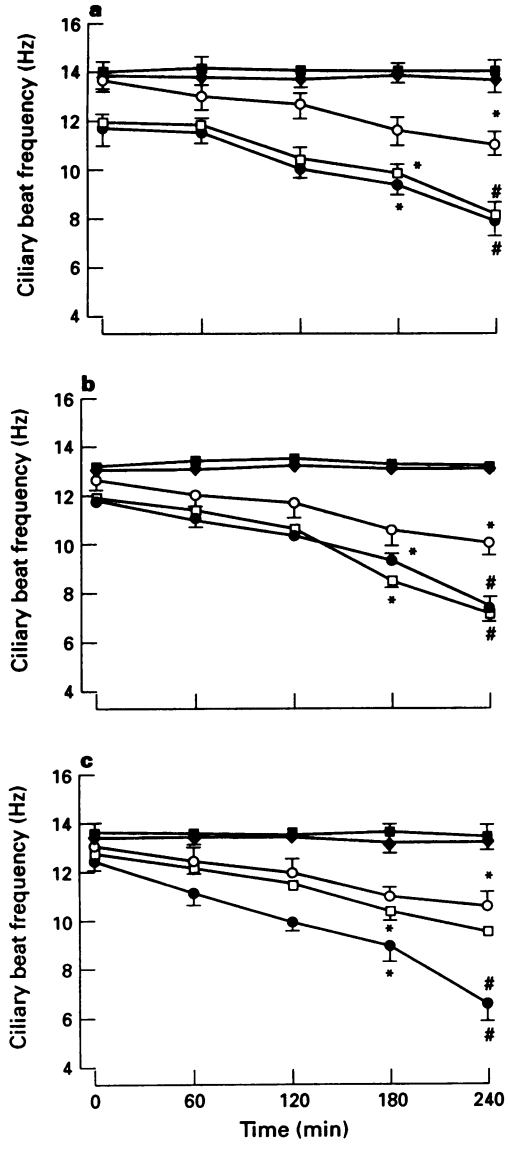

Figure 3 The effect of propranolol, ICI 118551 and atenolol on the protection afforded by salmeterol on pyocyanin-induced ciliary beat slowing. (a) Propranolol (10^{-7} M), (b) ICI 118551 (10^{-6} M) and (c) atenolol (10^{-6} M) alone (◆) had no effect on baseline ciliary beat frequency (CBF) compared to control CBF in medium 199 alone (■). All three β-antagonists (□) did significantly ($P<0.05$) reduce the protective effect of salmeterol (○) on pyocyanin-induced ciliary slowing (●). # = ciliary dyskinesia, * = epithelial disruption.

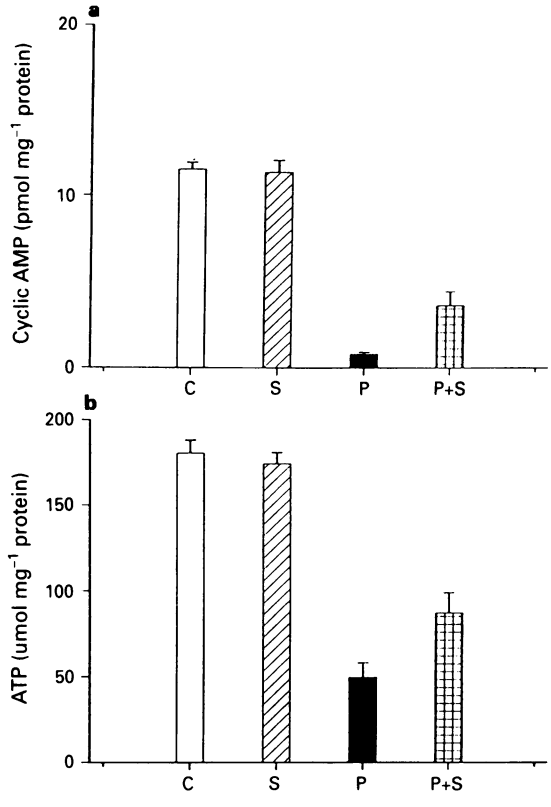

Figure 4 The effect of salmeterol on pyocyanin-induced reduction in intracellular cyclic AMP and ATP. Pyocyanin (P) produced a 93% reduction in cyclic AMP (a) and an 88% reduction in ATP (b) compared to controls (c) in medium 199 alone. Treatment of the cells with salmeterol (S) 2×10^{-7} M had no effect on basal levels of adenosine nucleotides but did significantly ($P<0.05$) protect (P + S) by 26% and 29% respectively against the reduction produced by pyocyanin in intracellular cyclic AMP and ATP.

reduces mucociliary clearance (Wilson *et al.*, 1987; Munro *et al.*, 1989; Kanthakumar *et al.*, 1993). Pyocyanin also enhances the oxidative metabolism of neutrophils (Ras *et al.*, 1990), inhibits epithelial cell growth (Cruickshank *et al.*, 1953) and affects lymphocytes function (Nutman *et al.*, 1987). Pyocyanin is found in the sputum of colonized patients at concentrations as high as 27 μg ml^{-1} (Wilson *et al.*, 1988) which is sufficient to cause all these *in vitro* effects.

Pyocyanin (20 μg ml^{-1}) caused CBF to slow by >50% during 4 h, and this was associated with epithelial disruption after 3 h and ciliary dyskinesia after 4 h. Salmeterol reduced the effects of pyocyanin on epithelial cells. The concentrations of salmeterol that were studied were chosen because 2×10^{-7} M salbutamol just provides maximum stimulation of β_2-adrenoceptors in a range of tissues (Coleman & Nials, 1989; Ball *et al.*, 1991). In the presence of salmeterol there was less ciliary slowing, no ciliary dyskinesia and the onset of epithelial disruption was delayed. The interaction of salmeterol with the epithelium was such that the drug could be removed by washing after 30 min without affecting its action. This suggests that the drug persists in the tissue after this time, and may result from its lipid solubility (Bradshaw *et al.*, 1987; Nials *et al.*, 1993). These data are consistent with the studies of Johnson (1990) on guinea-pig smooth muscle and Devalia *et al.* (1992) on human cultured bronchial epithelial cells. Previous studies using animal tracheal tissue have shown CBF to increase with β-agonists at similar concentrations to those used in the present study (Verdugo *et al.*, 1980; Wong *et al.*, 1988). In our studies, 2×10^{-7} M salmeterol had no effect on baseline CBF, nor intracellular cyclic AMP and ATP levels. This difference is likely to be related to the species studied, or possibly the site from which the epithelial cells were obtained. The effects of β-agonists on human CBF may be concentration-dependent because high concentrations of salmeterol (10^{-6} M) have been shown to stimulate an approximate 10% increase in CBF of human cultured bronchial epithelial cells. This was associated with an approximate 10 fold increase in intracellular cyclic AMP levels (Devalia *et al.*, 1992).

The non-specific β-receptor antagonist, propranolol and the β_2-selective receptor antagonist, ICI 118551 (Nials *et al.*, 1993) both completely abolished the action of salmeterol on pyocyanin-induced ciliary slowing, ciliary dyskinesia and epithelial disruption. Our findings on the effect of propranolol are in agreement with those of Ball *et al.* (1991) who suggested from their studies of guinea-pig trachea that a simple competitive interaction existed between the two com-

pounds. The selective β_1-receptor antagonist, atenolol had only a small effect on the action of salmeterol on pyocyanin-induced ciliary slowing, and had no effect on either ciliary dyskinesia or epithelial disruption. The effects of atenolol could be ascribed to its weak β_2-antagonist activity (Nials *et al.*, 1993). Isoprenaline, which has short-acting β_2-adrenoceptor agonist activity, was also able to inhibit pyocyanin-induced ciliary slowing, ciliary dyskinesia and epithelial disruption, although, as in other studies (Nials *et al.*, 1993), it was less potent than salmeterol.

We have previously shown that ciliary beat slowing by pyocyanin is associated with a fall in intracellular cyclic AMP and ATP, and is prevented by the cyclic AMP analogue, dibutyryl cyclic AMP, the phosphodiesterase inhibitor isobutyl methylxanthine, and the adenylate cyclase stimulant, forskolin (Kanthakumar *et al.*, 1993). Salmeterol protected against the pyocyanin-induced fall in intracellular adenosine nucleotides, and this was associated with a reduction in pyocyanin-induced ciliary slowing, which suggests that these events could be mediated via a common mechanism. As ATP is an essential energy source for beating cilia (Satir, 1989; Satir & Sleigh, 1990), it is possible that the effects of pyocyanin on CBF and coordination of ciliary beat are directly mediated through the fall in intracellular ATP levels. Lansley and colleagues (1992) have proposed that the concentration of intracellular cyclic AMP may affect the availability or utilisation of ATP by ciliary axonemes. β_2-Adrenoceptor agonists have been shown to increase cyclic AMP levels in both animal and human respiratory epithelial cells (Lansley *et al.*, 1992; Devalia *et al.*, 1992) and this is likely, therefore, to explain the effect of salmeterol on pyocyanin-induced ciliary beat slowing. In our previous studies pyocyanin-induced ciliary slowing after 2 h was completely reversed after washing, and the associated fall in adenosine nucleotides at 2 h occurred at a time when the epithelial cells were not damaged as assessed by lactate dehydrogenase release and trypan blue exclusion. These observations suggest that cell damage is not the primary cause of slowed CBF, and reduced levels of cyclic AMP and ATP (Kanthakumar *et al.*, 1993). Agents which raise intracellular cyclic AMP, including salmeterol in the present study, also prevent pyocyanin-induced disruption of the integrity of the epithelial surface.

Salmeterol may benefit patients colonised by *P. aeruginosa*, not only by its bronchodilator action, but by protecting epithelial cells from pyocyanin-induced slowing and disorganisation of CBF, disruption of epithelial integrity, and the consequent reduction of mucociliary transport (Wilson, 1988; Yeates *et al.*, 1976).

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