INFECTION AND IMMUNITY, July 1993, p. 2848–2853
0019-9567/93/072848-06$02.00/0

Vol. 61, No. 7

Mechanisms of Action of *Pseudomonas aeruginosa* Pyocyanin on Human Ciliary Beat In Vitro

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Received 1 April 1993/Accepted 20 April 1993

Pyocyanin is a blue redox active pigment produced by *Pseudomonas aeruginosa*. It is present at concentrations of up to 10^{-4} M in sputa from patients with cystic fibrosis and bronchiectasis who are heavily colonized with this organism. Pyocyanin, at physiologically relevant concentrations, slows human nasal ciliary beat frequency (CBF) in vitro and leads to disruption of the epithelium. Pyocyanin-induced slowing of CBF after 2 h was associated with a significant fall in intracellular cyclic AMP (cAMP) (90%) and ATP (66%) and was reversible after the pyocyanin was removed by washing. These effects were not mediated through interaction with neutrophils. The pyocyanin-induced fall in CBF was not affected by EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], pyrazinamide, 8-phenyltheophylline, indomethacin, or antioxidants, including catalase (500 U/ml), superoxide dismutase, and *N*-acetylcysteine. Ciliary slowing was, however, prevented (>70%) by isobutylmethylxanthine and forskolin, both of which increase intracellular cAMP, and also by the cAMP analog, dibutyryl cAMP. There was also a concomitant protection against the fall in both cAMP and ATP. These agents also delayed the onset of epithelial disruption associated with pyocyanin treatment. In contrast, treatment with the iron chelator desferrioxamine prevented epithelial disruption, although it had no effect on pyocyanin-induced slowing of CBF. It appears that ciliary slowing can be dissociated from epithelial disruption and that the effects of pyocyanin on CBF are associated with a fall in both intracellular cAMP and ATP.

Pseudomonas aeruginosa frequently infects the bronchial trees of patients with cystic fibrosis and other forms of bronchiectasis. Once colonization is established, the bacterium is rarely eliminated, despite antibiotic treatment (10, 16). *P. aeruginosa* causes an exuberant chronic host inflammatory response which together with bacterial products damages host tissue, leading to clinical deterioration and worsening lung function (1, 3, 9, 10, 16, 23).

The phenazine pigment pyocyanin is one of the factors produced by *P. aeruginosa* which contribute to the pathogenicity of the organism. It is found in the sputa of colonized patients at concentrations as high as 27 μg/ml (>10^{-4} M) (40). Pyocyanin is a redox compound (15) which has been previously shown to stimulate redox cycling in bacteria, liver cells, and a human epithelial cell line (2, 5, 14). Free-radical generation has been previously proposed as the mechanism of its antibacterial action (14). Pyocyanin perturbs human respiratory epithelium, causing a fall in ciliary beat frequency (CBF) and a disruption of epithelial integrity (38, 39), and slows tracheal mucociliary transport in the guinea pig in vivo (22). Pyocyanin also enhances the oxidative metabolism of neutrophils (26), and it has been suggested previously that the action of pyocyanin on ciliary beating might be via the activation of mucosal neutrophils and generation of toxic oxygen radicals (17). Using a battery of pharmacological interventions, we have investigated the mechanism of action of pyocyanin on CBF and epithelial disruption of freshly obtained human nasal epithelial cells and epithelial cell culture monolayers.

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MATERIALS AND METHODS

Preparation of pyocyanin. Pyocyanin was prepared by photolysis of phenazine methosulfate (18) (Aldrich Chemical, Milwaukee, Wis.) and purified and characterized as previously reported (40).

Pharmacological agents. The pharmacological agents were chosen because of the previously reported actions of pyocyanin or known mechanisms of ciliary beat slowing or for their effects on intracellular cyclic AMP (cAMP). EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] is a calcium chelator (33); catalase, superoxide dismutase, *N*-acetylcysteine, and the iron chelator desferrioxamine are involved in oxidant defense (7, 8, 11, 32); L-*N*-methyl arginine inhibits the production of nitric oxide (37); indomethacin is a cyclooxygenase inhibitor (34); pyrazinamide and 3-aminobenzamide inhibit poly(ADP)-ribosyltransferases (25, 31); dibutyryl cAMP, 3-isobutylmethylxanthine (IBMX), and forskolin influence intracellular cAMP (6, 20, 27, 35); and 8-phenyltheophylline is an adenosine receptor antagonist (36). Pharmacological agents were obtained from Sigma Chemical Co. (Dorset, United Kingdom) and were dissolved in medium 199 (unless otherwise indicated) prior to being added to the cell suspension. In separate experiments (data not shown), each pharmacological agent was itself shown not to affect CBF or the integrity of the epithelium under the conditions of the experimental protocol. The epithelial cells were treated with the pharmacological agent for 15 min (unless otherwise indicated) prior to the addition of pyocyanin, and the slide preparation was then constructed. The series of experiments with EGTA were carried out in calcium-free minimal essential medium (Joklik's modified; GIBCO, Paisley, United Kingdom), and the pretreatment of epithelial cells was for 1

h. The series of experiments with desferrioxamine were carried out in iron-free Hanks balanced salt solution (GIBCO), and the pretreatment of epithelial cells was for 1 h.

Human nasal epithelium. Strips of normal human nasal ciliated epithelium were obtained from the inferior turbinate with a cytology brush (12, 28, 38–40) and dispersed by agitation in cell culture medium 199 with Earle's salts and HEPES (*N*-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (Flow Laboratories, Inc., McLean, Va.). The sample of epithelium was divided, and sealed microscope coverslip-slide preparations were constructed for assessment by light microscopy. Ciliary beating was measured photometrically from 10 marked sites at 37°C as previously described (38–40). Ciliary dyskinesia, ciliostasis, and disruption of the integrity of the epithelial surface (irregularity and breakup of the previously smooth intact surface) were recorded when present. A recording of 0 CBF was made only when ciliary beating had ceased on the whole epithelial strip.

Cell culture monolayers. Cell culture monolayers (free from neutrophils) were established from human bronchial explants on Falcon tissue culture plastic (Becton Dickinson, Oxford, United Kingdom) in 1:1 Dulbecco's modified Eagle's medium-Ham's F12 cell culture medium (GIBCO) with 2% low-protein serum replacement (Sigma Chemical Co.) and 50 IU of penicillin per ml and 50 μg of streptomycin per ml at 37°C in 5% CO_2. The medium was changed every other day. The explant was removed as soon as a ciliated outgrowth monolayer with a diameter of 20 to 30 mm had been established. On removal of the explant, the medium was changed to medium 199 without antibiotics after 2 washes with medium 199 and incubated at 37°C for 24 h to equilibrate before commencement of the experiment. Ten well-separated areas of ciliated cells were randomly chosen, and their positions were marked. Measurements of CBF were made photometrically at intervals.

Experimental protocols. For each experiment with nasal epithelial strips, three sealed microscope coverslip-slide preparations containing epithelium from one volunteer plus medium 199 alone, medium 199 plus 10^{-4} M pyocyanin (20 μg/ml), or medium 199 plus 10^{-4} M pyocyanin plus a pharmacologic agent were made. Six such experiments were performed for each pharmacologic agent, and CBF was measured for 4 h. For experiments to determine the reversibility of the pyocyanin effect on CBF, a sample of nasal epithelium was divided equally and the CBF was measured in each half. Pyocyanin (10^{-4} M) was then added to one sample for 2 h before CBF was again recorded. Cells were then washed twice in medium 199 by centrifugation and resuspended in medium 199. CBF was then measured for 4 h (n = 6). For each experiment with cell culture monolayers, CBF was measured on two occasions 24 h apart before the medium 199 bathing the monolayer was replaced by medium 199 alone, medium 199 plus 10^{-4} M pyocyanin, or medium 199 plus 10^{-4} M pyocyanin plus a pharmacological agent. These experiments were continued until complete ciliostasis had occurred in the 10 groups of cells chosen for measurement of CBF. Six experiments were performed for each pharmacological agent.

Effect of pyocyanin and neutrophils on nasal epithelium. Neutrophils were obtained by dextran sedimentation followed by Ficoll gradient separation by the method described by Boyum (4). The final concentration of neutrophils in medium 199 was 10^6/ml. The following four slide preparations containing nasal epithelium were constructed for each of six experiments: medium 199 alone, medium 199 plus neutrophils, medium 199 plus 10^{-4} M pyocyanin, and medium 199 plus neutrophils plus 10^{-4} M pyocyanin. The usual experimental protocol was then followed.

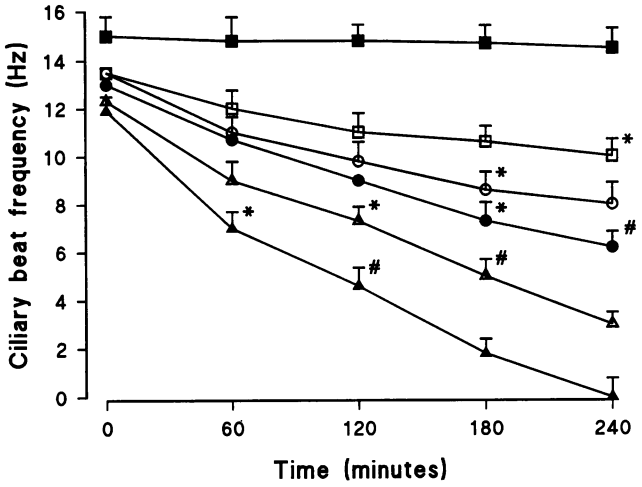

FIG. 1. Effect of pyocyanin on human nasal CBF. Control medium 199 (■) and pyocyanin at 5 (□), 10 (○), 20 (10^{-4} M) (●), 30 (△), and 40 (▲) μg/ml were used. Results are given as means ± SEM of six experiments. Epithelial disruption (*) and ciliary dyskinesia (#) are indicated at the time that they were first observed.

ATP and cAMP measurement. Levels of ATP in nasal epithelium were measured with a quantitative spectrophotometric kit (Sigma Chemical Co.). cAMP levels were measured with an enzyme immunoassay kit (Amersham International, Amersham, United Kingdom). Nasal epithelial samples (n = 6) were treated with 10^{-4} M pyocyanin in medium 199 for 2 h in the presence or absence of forskolin or IBMX (cAMP assay) and in the presence or absence of dibutyryl cAMP, IBMX, or forskolin (ATP assay). The concentrations of agents used were as described previously. Cellular protein levels were assessed by the method of Lowry et al. (21), and levels of adenosine nucleotides were expressed as per milligram of cellular protein.

Statistical analysis. Statistical analysis was carried out by using the Wilcoxon signed rank paired test. Data are expressed as means ± standard errors of the mean (SEM). The mean percent inhibition of pyocyanin-induced effects (protection) afforded by the various pharmacological agents was calculated as 100 × [mean (n = 6) CBF of pyocyanin with inhibitor − mean (n = 6) CBF of pyocyanin without inhibitor]/[mean (n = 6) CBF of control − mean (n = 6) CBF of pyocyanin without inhibitor].

RESULTS

Pyocyanin caused a progressive and concentration-dependent slowing of human nasal ciliary beating (Fig. 1). Pyocyanin at concentrations which have been observed in sputum (10^{-4} M) caused a fall to 42% of the control by 4 h; this concentration caused a disruption of epithelium integrity by 3 h and ciliary dyskinesia by 4 h. This concentration, which was used for all further experiments, also caused a marked fall in both intracellular cAMP (90%) and ATP (66%) at 2 h (Fig. 2); there was little change in these levels following a further 2-h incubation with the pigment (data not shown). After 2 h of incubation with pyocyanin, the epithelium was intact as determined by light microscopy, there was no leakage of lactate dehydrogenase or ATP into the supernatant (data not shown), and the cells were impervious to

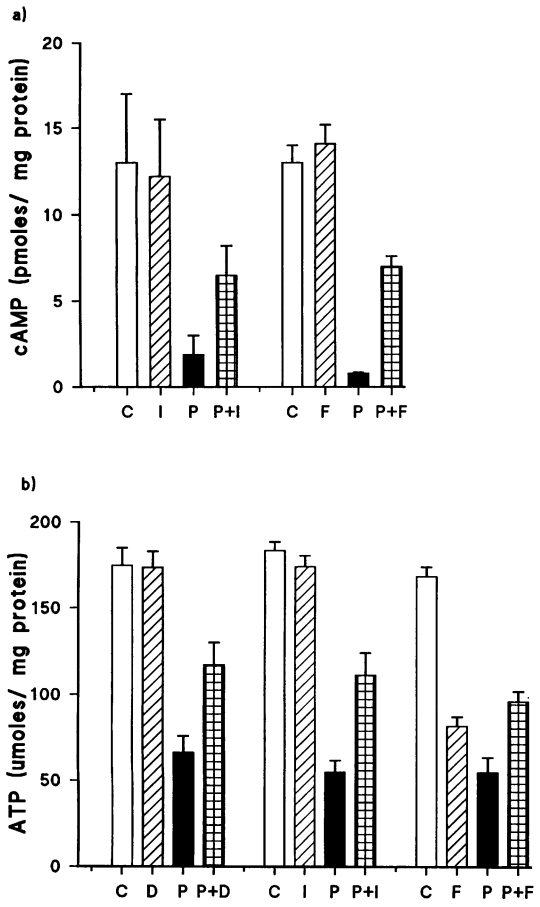

FIG. 2. Effect of incubation with pyocyanin for 2 h on intracellular levels of cAMP and ATP and effects of forskolin, IBMX, or dibutyryl cAMP. Pyocyanin causes a concomitant fall in cAMP and ATP levels in human nasal epithelium. (a) Effects of forskolin (F) and IBMX (I) on pyocyanin-induced lowering of cAMP. (b) Effects of forskolin, IBMX, and dibutyryl cAMP (D) on pyocyanin-induced lowering of ATP. Control cells (C) were incubated with medium 199 alone. Results are given as means ± SEM of six experiments.

trypan blue, suggesting that epithelial damage occurs subsequent to the initial fall in cAMP and ATP. Treatment of nasal brushings with pyocyanin (10^{-4} M) for 2 h resulted in a 21.5% ± 1.8% fall in CBF. Washing the cells to remove pyocyanin resulted in a rapid recovery of CBF which made it indistinguishable from control CBF after 4 h. Measurements of CBF for test cells were 13.1 ± 0.1 Hz before exposure to pyocyanin, 10.2 ± 0.3 Hz after 2 h of exposure to pyocyanin, 12.0 ± 0.2 Hz immediately after washing, and 12.2 ± 0.1 Hz 4 h after washing, and those for control cells were 13.1 ± 0.1, 13.0 ± 0.1, 13.2 ± 0.1, and 12.5 ± 0.1 Hz at the same time points. It was also possible to dissociate CBF slowing from the epithelial damage observed at 4 h with desferrioxamine. The iron chelator desferrioxamine had no effect on pyocyanin-induced slowing of CBF but prevented pyocyanin-induced epithelial disruption (data not shown). Partial protection against CBF slowing was achieved with high-dose catalase (5,000 U/ml) and 3-aminobenzamide (5 mM) (29 and 27%, respectively, at 4 h), although neither reagent prevented epithelial disruption.

These effects of pyocyanin on CBF and the disruption of nasal epithelium were unaffected by a number of pharmacological agents, including a calcium chelator, three separate antioxidants, an adenosine receptor antagonist, and inhibitors of cyclooxygenase and poly(ADP) ribosyltransferase (Table 1); all reagents were used at pharmacologically active concentrations. Pyocyanin is known to inhibit the action of nitric oxide. The nitric oxide synthetase inhibitor (L-*N*-methyl arginine) had no effect on basal CBF (data not shown), suggesting that the effect of pyocyanin on CBF was not mediated through an alteration in nitric oxide production.

TABLE 1. Pharmacological agents with no effect on pyocyanin-induced ciliary slowing

Pharmacological agent (concn)	CBF of epithelium in[a]: Medium 199 alone	Medium 199 + pyocyanin	Medium 199 + pyocyanin + pharmacological agent
Catalase (500 U/ml)	13.0 ± 0.2	7.0 ± 0.3	7.2 ± 0.2
Superoxide dismutase (2,000 U/ml)	13.9 ± 0.3	8.0 ± 0.4	7.8 ± 0.3
Superoxide dismutase (85 U/ml)	12.2 ± 0.6	5.8 ± 0.5	5.4 ± 0.6
N-Acetylcysteine (100 μg/ml)	12.8 ± 0.7	6.0 ± 0.4	5.8 ± 0.4
Indomethacin (3 mM)	14.8 ± 0.2	7.0 ± 0.3	7.6 ± 1.2
Desferrioxamine (20 mM)	15.1 ± 0.4	4.1 ± 1.6	5.0 ± 0.8
EGTA (1 mM)	13.2 ± 0.3	6.0 ± 0.4	5.8 ± 0.6
Pyrazinamide (150 μM)	13.8 ± 0.4	6.5 ± 0.3	6.3 ± 0.4
8-Phenyltheophylline (100 μM)	13.7 ± 0.1	8.2 ± 0.2	8.0 ± 0.3

[a] Data are means ± SEM. All results are for six experiments, and CBF was measured for 4 h.

Pyocyanin-induced slowing of CBF was significantly prevented by the adenylate cyclase activator forskolin (0.75 mM; 74% protection at 4 h, $P < 0.05$) and the phosphodiesterase inhibitor IBMX (1 mM; 78% protection at 4 h, $P < 0.05$). The cAMP analog dibutyryl cAMP (300 μM) also prevented ciliary slowing caused by pyocyanin (84% protection, $P < 0.05$) (Fig. 3). In each case, the onset of epithelial disruption caused by pyocyanin was delayed, its extent was reduced, and ciliary dyskinesia was not observed. Both forskolin and IBMX at the same concentrations protected against the fall in cAMP (51 and 41% protection, respectively) and ATP (36 and 44%, respectively) levels (Fig. 2). Dibutyryl cAMP also protected against the fall in intracellular ATP (47% protection). At the concentrations used, these reagents did not cause an increase in basal CBF. IBMX did not affect basal intracellular cAMP and ATP levels, and dibutyryl cAMP did not affect basal ATP levels, but forskolin caused a fall in basal ATP levels (without affecting CBF) and a small rise in basal cAMP levels.

The addition of neutrophils to preparations of nasal brushings from the same subject caused a small fall in CBF from 13.8 ± 0.6 to 12.8 ± 0.8 Hz at 4 h. Neutrophils did not affect the integrity of the tissue. In similar preparations, 10^{-4} M pyocyanin added together with neutrophils had a greater effect on CBF (reducing it to 8.6 ± 0.9 Hz at 4 h) than pyocyanin alone (9.4 ± 0.4 Hz). The effects of pyocyanin and neutrophils were additive rather than synergistic (Fig. 4). A neutrophil-free ciliated human bronchial epithelial cell culture monolayer was also employed. This preparation behaved in a manner similar to nasal brushings. Pyocyanin caused a slowing of CBF, which was partially reversed by dibutyryl cAMP, and the time that ciliary beating continued

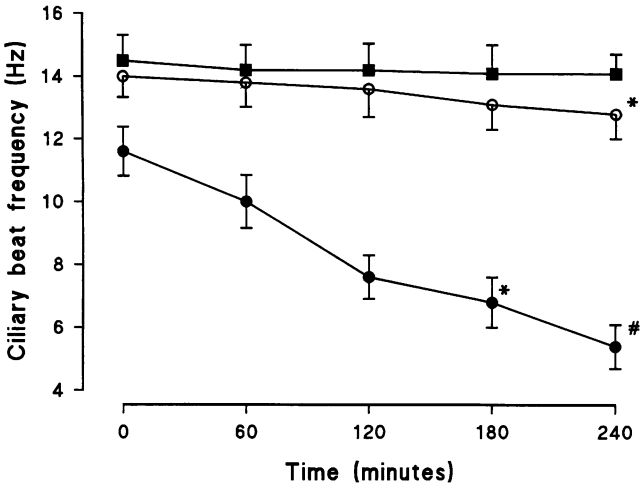

FIG. 3. Effect of dibutyryl cAMP on pyocyanin-induced slowing of nasal CBF. Control medium 199 (■), 10^{-4} M pyocyanin (●), and 10^{-4} M pyocyanin plus dibutyryl cAMP (○) were used. Results are given as means ± SEM of six experiments. Epithelial disruption (*) and ciliary dyskinesia (#) are indicated at the time that they were first observed. Similar degrees of protection were achieved with forskolin and IBMX (see the text).

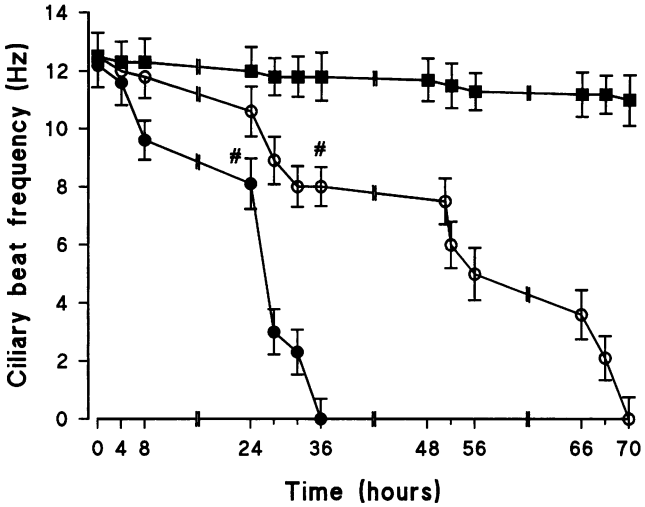

FIG. 5. Effect of pyocyanin on human bronchial epithelial cell culture monolayers. Control medium 199 (■), 10^{-4} M pyocyanin (●), and 10^{-4} M pyocyanin plus dibutyryl cAMP (○) were used. As with nasal brushings, pyocyanin slows CBF, and this effect is partially reversed by dibutyryl cAMP. Results are given as means ± SEM of six experiments. Ciliary dyskinesia (#) is indicated at the time that it was first observed.

was prolonged by dibutyryl cAMP (Fig. 5). These data suggest that neutrophils are not involved in the effects of pyocyanin on human CBF.

DISCUSSION

Pyocyanin is produced by *P. aeruginosa* and may facilitate the persistence of the organism in colonized airways by slowing ciliary beating and reducing mucociliary clearance (22, 24, 38, 39). A number of pharmacological interventions were used to delineate the mode of action of pyocyanin.

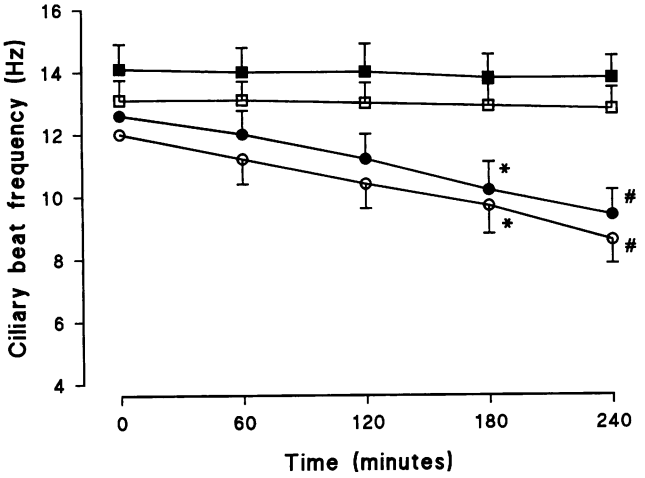

FIG. 4. Effect of pyocyanin on CBF of human nasal epithelium with and without addition of neutrophils. Control medium 199 (■), 10^{-4} M pyocyanin (●), 10^6 neutrophils per ml (□), and 10^{-4} M pyocyanin plus 10^6 neutrophils per ml (○) were used. The effects of pyocyanin and neutrophils were additive. Results are given as means ± SEM of six experiments. Epithelial disruption (*) and ciliary dyskinesia (#) are indicated at the time that they were first observed.

Antioxidants and inhibitors of cyclooxygenase had no effect on either CBF slowing or the disruption of epithelium integrity. Negative data must, however, be treated with some caution, since it is difficult to demonstrate that these agents were active at the site of pyocyanin-induced changes. Our data do contradict earlier reports that neutrophils are required for pyocyanin-induced slowing of ciliary beating (17). High concentrations of catalase (5,000 U/ml) did afford some protection against CBF slowing but no protection against epithelial disruption. These data suggest that hydrogen peroxide generated intracellularly may leak from the cell onto the epithelial surface and could be involved in pyocyanin action on ciliary beating.

Pyocyanin treatment leads to a fall in intracellular ATP. As ATP is an essential energy source for beating cilia (29, 30), it is possible that the effects of pyocyanin are directly mediated through a fall in ATP levels. Pyocyanin undergoes intracellular substrate cycling with the formation of superoxide and loss of NADH (13). A loss of reducing power will cause a fall in ATP levels. In the presence of iron, superoxide is converted to the highly reactive hydroxyl radical (8, 11), which can cause damage to membranes and DNA, again with a consequent fall in ATP. DNA damage is unlikely as the cause of the fall in ATP, as inhibition of the DNA repair enzyme poly(ADP)-ribosyltransferase (which consumes ATP) by pyrazinamide has no effect on pyocyanin-induced slowing of CBF. 3-Aminobenzamide also inhibits poly(ADP) ribosyl transferase, but its protective effect in our experiments is probably due to its known weak inhibitory action on phosphodiesterases (31).

There is a clear association between pyocanin-induced CBF slowing and a fall in both intracellular ATP and cAMP. This occurs at a time when the cells are not obviously damaged and the effect of pyocyanin on CBF can be reversed by washing. It was also possible to dissociate the pyocyanin-induced epithelial damage from changes in CBF by the use of the iron chelator desferrioxamine. These

results suggest that cell damage is not the primary cause of reduced levels of cAMP and ATP and lowered CBF.

The effects of pyocyanin on CBF are partially reversed by the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor IBMX. Both of these agents are known to raise cAMP levels by different mechanisms, and both partially protect against the pyocyanin-induced fall in ATP and cAMP. It is unlikely that these agents are acting through other, non-cAMP-related pathways (20, 27, 35), as the stable cAMP analog, dibutyryl cAMP, offers similar protection against pyocyanin. Thus, it appears that alterations in the levels of cAMP are associated with changes in ciliary beating. At the concentrations used in our study, these agents did not alter basal levels of cAMP or CBF, although forskolin did cause a fall in basal levels of ATP without any change in CBF. Our data are in agreement with those of Di Benedetto and colleagues (6), who showed that dibutyryl cAMP (10^{-4} M) had no effect on human nasal ciliary beating, although 10-fold-higher concentrations did cause a small (approximately 10%) increase in CBF. High concentrations of forskolin (10^{-3} M) also stimulate basal CBF (approximate 23% increase) in the rabbit (35), although this was associated with a nonphysiological (approximately 20-fold) increase in intracellular cAMP. The fall in intracellular cAMP in response to pyocyanin cannot be related simply to the concomitant fall in its precursor ATP, since three unrelated agents (forskolin, IBMX, and dibutyryl cAMP) all cause parallel increases in both ATP and cAMP in cells treated with pyocyanin. These data suggest that cAMP can protect against the pyocyanin-induced fall of ATP. This may be a direct effect caused by stimulation of ATP production (19, 35) within cells treated with pyocyanin, or it could occur indirectly through an unknown mechanism. The disruption of epithelial integrity was also delayed by agents that raised intracellular cAMP. This might also occur by stimulation of ATP production or by an unknown mechanism protecting against cell damage.

In conclusion, we have demonstrated that the effect of pyocyanin on slowing of CBF is associated with a fall in both intracellular cAMP and ATP and can be dissociated from cell damage. It appears that the controlling factor in these events is the concentration of intracellular cAMP. This is in agreement with the recent report by Lansley and colleagues, who suggested that cAMP may affect the availability or use of ATP by the ciliary axoneme (19).

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