The Effect of Rolipram, a Type IV Phosphodiesterase Inhibitor, on Pseudomonas aeruginosa Infection of Respiratory Mucosa

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ABSTRACT
We have investigated the effect of rolipram, a type IV phosphodiesterase inhibitor, on Pseudomonas aeruginosa infection of the respiratory mucosa of an organ culture model and on the reduction in intracellular cAMP levels seen in human nasal epithelial cells incubated with P. aeruginosa culture filtrate. We have compared rolipram with salmeterol, a long-acting beta-2 agonist, and have also studied the effect of the two agents together. Infected organ cultures had significantly (P ≤ .05) increased epithelial damage. Rolipram significantly (P ≤ .05) reduced P. aeruginosa-induced epithelial damage and reduced the total number of bacteria adhering to the respiratory mucosa (P ≤ .04) in a concentration-dependent manner, although neither rolipram nor salmeterol affected P. aeruginosa growth in broth cultures. Rolipram reduced P. aeruginosa-induced mucosal damage more than salmeterol (P ≤ .03). The effect of the two agents was neither additive nor synergistic. Rolipram, salmeterol and both agents together significantly (P ≤ .01) increased intracellular cAMP levels in epithelial cells treated with P. aeruginosa culture filtrate. Rolipram alone increased cAMP more than salmeterol or both agents together (P ≤ .01), probably because of an interaction between the two agents. These results suggest that agents that elevate intracellular cAMP protect the epithelium during bacterial infection. Rolipram is more effective than salmeterol in preventing P. aeruginosa-induced epithelial damage.

P. aeruginosa is an opportunistic pathogen that frequently colonizes the respiratory tract of patients with cystic fibrosis, bronchiectasis and severe chronic bronchitis (Fick, 1989; Pitt 1986). It is particularly associated with progressive and ultimately fatal chronic respiratory infection in cystic fibrosis. Management of patients with chronic bronchial infection includes regular postural physiotherapy and antibiotic therapy for exacerbations (Rayner et al., 1994a). However, once P. aeruginosa infection is established, it is rarely eliminated despite intensive antibiotic therapy (Fick, 1989). Chronic P. aeruginosa infection stimulates an exuberant host inflammatory response (Pier, 1985), and this may be associated with deterioration in lung function, increased morbidity and mortality and impaired quality of life (Fick, 1989; Pitt, 1986; Rayner et al., 1994a). Patients infected with P. aeruginosa often require frequent or even continuous antibiotic therapy in order to suppress the numbers of bacteria in the lung (Hodson et al., 1981; Rayner et al., 1994b), which in turn reduces the level of inflammation. However, long courses of antibiotics may be poorly tolerated, and bacterial resistance commonly occurs (Wilson and Tsang, 1994). It seems unlikely that new antibiotics will improve this outcome, so preventive and adjunct therapies are important. Oral corticosteroids (Auerbach et al., 1985), nonsteroidal anti-inflammatory agents (Llewellyn-Jones et al., 1995), mucolytics (Shak et al., 1990) and immunization (Schaad et al., 1991) are currently under investigation.

Numerous studies have demonstrated that bacterial products may contribute to the pathogenesis of P. aeruginosa in the airway (Fick, 1989; Pitt, 1986; Pier, 1985). Pyocyanin is a blue phenazine pigment that is produced by P. aeruginosa and causes slowing of ciliary beat and disruption of the integrity of the epithelium in vitro (Wilson et al., 1987), as well as slowing of mucociliary transport in guinea pig trachea in vivo (Munro et al., 1989). Pyocyanin-induced ciliary slowing is associated with a decrease in both intracellular cAMP and ATP, and agents that raise intracellular cAMP inhibit the effect of pyocyanin on epithelium (Kanthakumar et al., 1993). Salmeterol is a long-acting beta-2 agonist (Nials et al., 1993; Anderson et al., 1994; Devalia et al., 1992) that reduces pyocyanin-induced declines in both intracellular cAMP and ATP that are associated with ciliary slowing and preserves CBF (Kanthakumar et al., 1994). Salmeterol also reduces the amount of epithelial damage that occurs in organ cultures infected with P. aeruginosa, and it reduces ultrastructural damage in nasal epithelial cells caused by the P. aeruginosa

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ABBREVIATIONS: PDE, phosphodiesterase; BHI, brain heart infusion; PBS, phosphate-buffered saline; MEM, minimal essential medium; BAL, bronchoalveolar lavage; IL-8, interleukin-8; TNF, tumor necrosis factor; IBMX, 3-isobutyl-1-methylxanthine.
toxins pyocyanin and elastase (Dowling et al., 1997). These data suggest that agents that elevate cAMP may protect respiratory epithelium from damage caused by bacterial infection.

We have now investigated the effect of rolipram, a type IV phosphodiesterase inhibitor, on *P. aeruginosa* infection of the respiratory mucosa *in vitro* and compared the effects of rolipram with those of salmeterol alone, an agent we have studied previously, and with the effects of both agents together. We have also assayed intracellular cAMP levels in human nasal epithelial cells treated with *P. aeruginosa* culture filtrate in the presence and absence of both agents separately and together.

### Materials and Methods

#### Bacteriology

*P. aeruginosa* strain P455 is a clinical isolate that has been previously studied in our laboratory (Wilson et al., 1987; Tsang et al., 1994; Dowling et al., 1997). P455 is a nonmucoid and pilated strain that produces alkaline protease, elastase, phenazine pigments, lipase, DNAase and rhamnolipid. P455 was stored at −70°C in a BHI broth (Oxoid, Basingstoke, UK) and glycerol mixture (Sigma, Poole, Dorset, UK) (80:20) and then retrieved onto BHI agar. After overnight culture, 2 to 3 colonies were dispersed in 5 ml of BHI broth and incubated overnight at 37°C with agitation. The culture was diluted with BHI to give an optical density of 0.365, which previous experiments had shown corresponded to approximately 1.0 × 10^6 cfu ml⁻¹. One milliliter of the culture was then washed twice through 10 ml of PBS (Oxoid). The bacterial pellet was resuspended in 1 ml of PBS, vortexed and viable counts performed.

#### Preparation of Salmeterol and Rolipram

Salmeterol hydroxynaphthoate (Glaxo Wellcome, Uxbridge, Middx, UK) 6.03 mg was dissolved in the minimal amount of glacial acetic acid and then diluted with PBS to give a concentration of 1 × 10⁻⁵ M. This was further diluted with MEM (Gibco, Paisley, UK) to give a final concentration of 4 × 10⁻⁷ M. Rolipram (Glaxo Wellcome) was dissolved in PBS to give a concentration of 9.5 × 10⁻⁶ M. This was further diluted with MEM to give final concentrations of 1 × 10⁻⁶ M, 5 × 10⁻⁷ M and 1 × 10⁻⁷ M.

#### Organ Cultures

This method has been described previously (Tsang et al., 1994; Jackson et al., 1996; Dowling et al., 1997). Briefly, human nasal turbinate tissue was resected from patients undergoing surgery for nasal obstruction and transported to the laboratory in MEM containing antibiotics (50 μg/ml streptomycin, 50 IU/ml penicillin and 50 μg/ml gentamicin). Dissection was performed in antibiotic medium to yield small squares approximately 3 mm² in area and 2 to 3 mm thick. The tissue was screened for ciliary activity in order to select tissue squares with at least one fully ciliated edge. The tissue was immersed in antibiotic medium for at least 4 hr in order to remove commensal bacteria and then immersed in non-antibiotic-containing medium for at least 1 h in order to remove the antibiotics.

A sterile Petri dish 3.5 cm in diameter (Sterillon, Stone, UK) was placed aseptically within a sterile Petri dish 6.0 cm in diameter. A strip of sterile filter paper (Whatman No. 1, Maidstone, UK) with dimensions approximately 5 mm by 70 mm was soaked in MEM without antibiotics and positioned aseptically across the diameter of the inner Petri dish. The filter paper strip adhered to the base of the inner Petri dish, and its edges were sealed with agar. Four milliliters of non-antibiotic-containing medium were pipetted into the outer petri dish. The filter paper strip acted as a wick to draw medium from the outer petri dish to the underside of the tissue.

#### Experimental Design

**Protocol 1: The effect of rolipram on *P. aeruginosa* infection of organ cultures.** For each experiment (n = 6), seven organ cultures were prepared: control, tissue infected with *P. aeruginosa* alone, tissue incubated with rolipram alone (5 × 10⁻⁷ M and 1 × 10⁻⁷ M) and tissue incubated with rolipram (1 × 10⁻⁶ M, 5 × 10⁻⁷ M and 1 × 10⁻⁷ M) and then infected with *P. aeruginosa*. Appropriate tissue squares were incubated with 4 ml of rolipram (1 × 10⁻⁶ M, 5 × 10⁻⁷ M and 1 × 10⁻⁷ M) for 30 min before assembly of the organ cultures. During this time, the other tissue squares were incubated with MEM alone.

**Protocol 2: Comparison of the effect of rolipram and salmeterol on *P. aeruginosa* infection of organ cultures.** For each experiment (n = 6), seven organ cultures were prepared: control, tissue infected with *P. aeruginosa* alone, tissue incubated with rolipram alone (1 × 10⁻⁶ M), tissue incubated with rolipram (1 × 10⁻⁶ M and salmeterol (4 × 10⁻⁷ M) together, and tissue incubated with rolipram (1 × 10⁻⁶ M), salmeterol (4 × 10⁻⁷ M) or rolipram and salmeterol together (same concentrations) and then infected with *P. aeruginosa*. A salmeterol-alone control was not performed because our previous study had shown no effect (Dowling et al., 1997). Appropriate tissue squares were incubated with 4 ml of rolipram (1 × 10⁻⁶ M), 4 ml of salmeterol (4 × 10⁻⁷ M), or 4 ml of rolipram and salmeterol together (same concentrations) for 30 min before assembly of the organ culture. During this time, the other tissue squares were incubated in MEM alone.

For both protocols, 20 μl of the washed bacterial suspension in PBS was gently pipetted onto the surface of the appropriate tissue squares immediately after organ culture construction, whereas the other organ cultures were inoculated with 20 μl of PBS. All organ cultures were incubated in a humidified atmosphere at 37°C in 5% CO₂ for 8 hr. At the end of each experiment, each of the four edges of the organ culture was touched with a sterile loop and plated onto BHI agar in order to assess the sterility of uninfected organ cultures and the purity of *P. aeruginosa* growth in infected organ cultures. The filter paper strip was then cut near the tissue with a sterile blade, removed with the tissue attached and fixed for scanning electron microscopy as previously described (Tsang et al., 1994; Jackson et al., 1996; Dowling et al., 1997).

#### Assessment of Tissue by Scanning Electron Microscopy

At the end of each experiment, tissue squares were given a coded number by an independent observer so that the original identity of the samples was unknown during analysis by scanning electron microscopy. Each tissue square was examined using an Hitachi S-4000 scanning electron microscope (Katsuta-shi, Ibaraki-Ken, Japan) by the same observer. The tissue was initially viewed at a magnification of ×50. A transparent acetate sheet with 100 equal squares was placed over the screen of the visual display unit. A predetermined pattern of 40 grid squares was selected for further viewing and analysis at ×3000 magnification. This pattern involved the horizontal axis, the vertical axis and two diagonal axes and yielded a representative survey of the mucosal surface measuring 1.42 × 10⁻⁶ μm². Care was taken to ensure that there was no overlap of squares in the center of the organ culture. Each square at a magnification of ×3000 was assessed using the same acetate sheet for percentage of the surface area occupied by four mucosal features: mucus, ciliated cells, unciliated cells and damaged epithelium. Excluding cells, cell debris, dead cells and loss of epithelium were scored together in the category of damaged epithelium. Unciliated areas were defined as areas not covered by cilia, with or without microvilli. Summation of the scores made it possible to assess the percentage of each field that was occupied by each mucosal feature.

The bacteria associated with each of the four mucosal features...
were counted. An approximation was made when large numbers of bacteria were present in sheets. In these instances, it was difficult to determine which mucosal component(s) the bacteria were adhering to, but observation of the tissue surrounding the bacteria enabled us to make an estimate. The total number of bacteria adhering to each organ culture was compared. In order to overcome the difficulty caused by different proportions of the organ culture surface being occupied by each mucosal feature, which made comparison between organ cultures difficult, we divided the total number of bacteria adhering to a mucosal feature by the proportion of the surface of the organ culture occupied by that feature (Rayner et al., 1995). This was referred to as the density of bacteria adherent to a mucosal feature.

**Effect of Rolipram and Salmeterol on *P. aeruginosa**

**Growth in BHI Broth**

*P. aeruginosa* (P455) was retrieved onto BHI agar. After overnight culture, three colonies were dispersed in 5 ml of BHI broth containing broth alone, rolipram (1 × 10^{-6} M), salmeterol (4 × 10^{-7} M) or rolipram and salmeterol together (same concentrations) and incubated at 37°C. Viable counts were performed by standard dilution methods on these cultures hourly over a period of 8 hr.

**Intracellular cAMP Assay**

In separate experiments, strips of human nasal ciliated epithelium were obtained with a cytology brush from both inferior turbinate of healthy volunteers (n = 6) who had been free of respiratory infection for at least 4 weeks (Wilson et al., 1987). This procedure has been approved by the Royal Brompton Hospital Ethics Committee. The strips were dispersed by gentle agitation of the brush in a total of 3.5 ml of cell culture medium 199 with Earle’s salts and HEPES (Flow Laboratories, Irvine, Scotland, UK). For each experiment, the nasal epithelium in medium 199 was equally divided among five vials: the three vials in which the epithelium had been incubated with broth alone, rolipram (1 × 10^{-6} M), salmeterol (4 × 10^{-7} M) or rolipram and salmeterol together (same concentrations) and incubated at 37°C. Viable counts were performed by standard dilution methods on these cultures hourly over a period of 8 hr.

**Bacteria**

The mean number [± standard deviation] of *P. aeruginosa* in 20 μl of PBS used for inoculation of organ cultures was (9.0 ± 2.6) × 10^6 cfu for protocol 1 and (3.5 ± 1.3) × 10^6 cfu for protocol 2. The inoculating dose was significantly (P ≤ .04) higher in protocol 1. At 8 hr all control organ cultures were sterile, and infected organ cultures gave a pure growth of *P. aeruginosa*.

**Scanning Electron Microscopy Analysis**

Control organ cultures at 8 hr exhibited very little mucosal damage. Neither rolipram (at all concentrations analyzed) nor rolipram and salmeterol together had a significant effect on any mucosal feature. *P. aeruginosa* infection caused a significant (P ≤ .05) increase in mucosal damage (tables 1 and 2; fig. 1A). Mucosal damage was observed as cell debris and dead or extruding cells, and there were areas in which the epithelium had been stripped away, exposing basal cells and collagen.

**Protocol 1.** Incubation of the tissue for 30 min with rolipram at the highest concentration significantly (P ≤ .05) reduced the amount of mucosal damage caused by *P. aeruginosa* infection. There were also significantly more ciliated cells present (P ≤ .05) (table 1). The lower concentrations of rolipram (5 × 10^{-7} M and 1 × 10^{-7} M) did not protect the epithelium against *P. aeruginosa*-induced mucosal damage. Organ cultures incubated with the lowest concentration of rolipram (1 × 10^{-7} M) had significantly (P ≤ .01) more mucosal damage and fewer ciliated cells after *P. aeruginosa* infection than those incubated with rolipram 1 × 10^{-6} M.

**Protocol 2.** Incubation of the tissue for 30 min with rolipram (1 × 10^{-6} M), salmeterol (4 × 10^{-7} M) or both agents together (same concentrations) before bacterial infection significantly (P ≤ .02) reduced the amount of mucosal damage and loss of ciliated cells caused by *P. aeruginosa* infection (table 2; fig. 1B). Tissue incubated with rolipram before bac-

**Statistics**

All comparisons of the mean percentage surface area occupied by each of the four mucosal features and the mean cAMP levels measured in nasal epithelium were analyzed using the Mann-Whitney U test. Total bacterial numbers and bacterial densities associated with each mucosal feature were analyzed using the Wilcoxon signed rank pairs test. P values ≤ .05 were judged to be significant.

**TABLE 1**

| Effect of rolipram on the damage caused by *Pseudomonas aeruginosa* infection of organ cultures |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Method                                        | Mucus                                        | Damaged Mucosa                                 | Ciliated Epithelium                           |
| control                                      | 8.2 ± 2.5                                     | 4.1 ± 0.8                                      | 22.4 ± 5.4                                    |
| rolipram (5 × 10^{-7} M)                      | 6.0 ± 4.0                                     | 4.5 ± 2.4                                      | 25.9 ± 6.4                                    |
| rolipram (1 × 10^{-7} M)                      | 8.6 ± 4.5                                     | 5.4 ± 2.5                                      | 24.7 ± 7.7                                    |
| *Pseudomonas* alone                          | 5.0 ± 2.7                                     | 43.6 ± 8.9*                                   | 16.0 ± 13.7                                   |
| *Pseudomonas* & roli. (10^{-6} M)             | 3.3 ± 0.7                                     | 43.6 ± 8.9*                                   | 16.0 ± 13.7                                   |
| *Pseudomonas* & roli. (5 × 10^{-7} M)         | 19.5 ± 13.5                                   | 43.6 ± 8.9*                                   | 16.0 ± 13.7                                   |
| *Pseudomonas* & roli. (10^{-7} M)             | 7.3 ± 2.8                                     | 55.6 ± 9.4*                                   | 16.0 ± 13.7                                   |

The results are expressed as the mean (n = 6) percent surface area of the organ culture occupied by each mucosal feature ± S.E.M.

*P ≤ .05 vs. control.

1*P ≤ .05 vs. *Pseudomonas* alone.

†*P ≤ .01 vs. *Pseudomonas* and rolipram 1 × 10^{-6} M.

‡*P ≤ .03 vs. *Pseudomonas* and rolipram 5 × 10^{-7} M.
Effect of salmeterol and rolipram on the damage caused by *Pseudomonas aeruginosa* infection of organ cultures

<table>
<thead>
<tr>
<th>Method Epithelium</th>
<th>Mucus</th>
<th>Damaged Mucosa</th>
<th>Ciliated Epithelium</th>
<th>Unciliated Epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>8.1 ± 3.1</td>
<td>6.2 ± 2.9</td>
<td>64.2 ± 7.9</td>
<td>21.5 ± 5.9</td>
</tr>
<tr>
<td>rolipram alone (10⁻⁶ M)</td>
<td>3.6 ± 2.0</td>
<td>5.4 ± 2.1</td>
<td>57.3 ± 13.8</td>
<td>33.7 ± 13.2</td>
</tr>
<tr>
<td>rolipram (10⁻⁶ M) &amp; salmeterol (4 × 10⁻⁷ M)</td>
<td>10.5 ± 4.2</td>
<td>4.8 ± 2.3</td>
<td>60.5 ± 9.5</td>
<td>24.2 ± 7.1</td>
</tr>
<tr>
<td><em>Pseudomonas</em> alone</td>
<td>14.7 ± 8.3</td>
<td>48.7 ± 6.7</td>
<td>5.1 ± 1.9</td>
<td>31.5 ± 8.4</td>
</tr>
<tr>
<td><em>Pseudomonas</em> &amp; rolipram (10⁻⁶ M)</td>
<td>4.2 ± 1.0</td>
<td>5.2 ± 1.2</td>
<td>58.2 ± 13.7</td>
<td>32.4 ± 13.4</td>
</tr>
<tr>
<td><em>Pseudomonas</em> &amp; salmeterol (4 × 10⁻⁷ M)</td>
<td>5.3 ± 1.9</td>
<td>15.9 ± 2.8</td>
<td>53.8 ± 9.5</td>
<td>25.0 ± 7.2</td>
</tr>
<tr>
<td><em>Pseudomonas</em> &amp; rolipram (10⁻⁶ M) &amp; salmeterol (4 × 10⁻⁷ M)</td>
<td>6.8 ± 3.9</td>
<td>13.6 ± 5.0</td>
<td>60.2 ± 11.6</td>
<td>19.4 ± 6.5</td>
</tr>
</tbody>
</table>

The results are expressed as the mean (n = 6) percent surface area of the organ culture occupied by each mucosal feature ± S.E.M.

*P* < .01 vs. control.
†*P* < .02 vs. *Pseudomonas* alone.
‡*P* < .03 vs. *Pseudomonas* and salmeterol.

**Fig. 1.** A) Electron micrograph of nasal turbinate tissue infected with *P. aeruginosa* showing bacteria adherent to mucus and damaged cells. Magnification ×4500; scale bar = 2.22 μM. B) Electron micrograph of nasal turbinate tissue pretreated with rolipram (1 × 10⁻⁶ M) and infected with *P. aeruginosa* showing mildly damaged mucosa with the bacteria adherent to damaged areas. Magnification ×4500; scale bar = 2.22 μM.

The main reason for the decline in the total number of bacteria adherent to the mucosa was the reduction in the percentage of damaged epithelium. Thus, although the density of bacteria adherent to damaged cells remained the same, the total number decreased because of the reduction in the percentage of damaged epithelium. Neither rolipram (10⁻⁶ M) nor salmeterol (4 × 10⁻⁷ M) nor the two agents together (same concentrations) affected the growth of *P. aeruginosa* in BHI broth culture (fig. 2).

**cAMP Levels in Human Nasal Epithelial Cells**

Incubation of nasal epithelium for 2 hr with *P. aeruginosa* culture filtrate significantly (P < .01) reduced the level of intracellular cAMP compared with control (fig. 3). Incubation with protocol 1. This may be related to the significantly (P ≤ .04) higher inoculating dose and subsequently the larger bacterial numbers present at the end of experiments in protocol 1. Incubation of the tissue with both rolipram and salmeterol before bacterial infection produced neither an additive nor a synergistic effect with respect to protection. The difference between rolipram alone and rolipram and salmeterol together was not statistically significant (P < .29).

**P. aeruginosa Adherence to Organ Cultures**

The interaction of *P. aeruginosa* with the organ cultures was similar to interactions previously reported (Plotkowski et al., 1991; Tsang et al., 1994). Bacteria were commonly seen adhering to mucus and damaged cells, particularly in the gaps between separated epithelial cells. *P. aeruginosa* sometimes grew in sheets, covering the surface of the tissue in a biofilm. Sheets of bacteria made counting bacteria difficult and also obscured the mucosal feature to which they were adherent. In these instances, an estimate of bacterial numbers was made that was probably an underestimate, and the mucosal feature to which the bacteria were adhering was judged at the edge of the sheet. Tissue incubated with rolipram (1 × 10⁻⁶ M) had significantly (P ≤ .04) lower total numbers of bacteria adherent to the mucosal surface than did tissue infected with *P. aeruginosa*. However, the lower concentrations of rolipram (5 × 10⁻⁷ M and 1 × 10⁻⁷ M) had no effect on the total number of bacteria compared with *P. aeruginosa* alone (table 3). Results similar to the effects of rolipram (10⁻⁶ M) were obtained with salmeterol (4 × 10⁻⁷ M) and with rolipram (1 × 10⁻⁶ M) and salmeterol (4 × 10⁻⁷ M) together (table 4). However, there was no change in the tropism of bacteria, which still adhered in similar density to damaged cells and mucus rather than to ciliated and unciliated cells (tables 3 and 4).

The main reason for the decline in the total number of bacteria adherent to the mucosa was the reduction in the percentage of damaged epithelium. Thus, although the density of bacteria adherent to damaged cells remained the same, the total number decreased because of the reduction in the percentage of damaged epithelium. Neither rolipram (10⁻⁶ M) nor salmeterol (4 × 10⁻⁷ M) nor the two agents together (same concentrations) affected the growth of *P. aeruginosa* in BHI broth culture (fig. 2).

The main reason for the decline in the total number of bacteria adherent to the mucosa was the reduction in the percentage of damaged epithelium. Thus, although the density of bacteria adherent to damaged cells remained the same, the total number decreased because of the reduction in the percentage of damaged epithelium. Neither rolipram (10⁻⁶ M) nor salmeterol (4 × 10⁻⁷ M) nor the two agents together (same concentrations) affected the growth of *P. aeruginosa* in BHI broth culture (fig. 2).
of nasal epithelium for 30 min with rolipram (1 × 10⁻⁶ M), salmeterol (4 × 10⁻⁷ M) or both agents together (same concentrations) before the addition of culture filtrate significantly (P ≤ .01) reduced the decline in intracellular cAMP. Rolipram had a significantly (P ≤ .01) greater effect on levels of intracellular cAMP than either salmeterol or both agents together (fig. 3).

**Discussion**

The effect of *P. aeruginosa* infection on the respiratory mucosa in this study was similar to that previously reported (Tsang et al., 1994; Dowling et al., 1997). *P. aeruginosa* infection caused epithelial damage that was associated with preferential loss of ciliated cells in most experiments. Bacteria adhered most frequently to damaged epithelial cells and mucus. Tropism of *P. aeruginosa* for mucus (Ramphal et al., 1987) and damaged epithelium (Plotkowski et al., 1991; Ramphal and Pyle, 1983) has been reported by a number of groups.

Rolipram is a PDE inhibitor that is selective for isoenzyme IV. Clinical studies on the effects of selective PDE inhibitors on respiratory function in humans are limited, but enoximone, a type III PDE inhibitor, decreased lung resistance and increased compliance in chronic obstructive pulmonary disease (Leeman et al., 1987). In addition, the administration of zardaverine, a selective PDE III and IV inhibitor, was found to have a modest but short-lasting bronchodilator effect in patients with reversible bronchial obstruction (Brunner et al., 1992). Underwood and colleagues (1994) demonstrated that rolipram (type IV) but not siguazodan (type III) inhibited antigen-induced contraction of guinea pig isolated trachea in vitro. In conscious guinea pigs, both zardaverine and the combination of rolipram and siguazodan were substantially more effective than rolipram or siguazodan alone.

**TABLE 3**

<table>
<thead>
<tr>
<th>Method</th>
<th>Mucus</th>
<th>Damaged Mucosa</th>
<th>Ciliated Epithelium</th>
<th>Unciliated Epithelium</th>
<th>Total No. of Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> alone</td>
<td>117.2 ± 52.1</td>
<td>201.8 ± 55.2</td>
<td>0.0</td>
<td>1.3 ± 0.6</td>
<td>3585 ± 1415</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> &amp; rolipram (10⁻⁶ M)</td>
<td>20.6 ± 13.9</td>
<td>86.3 ± 34.3</td>
<td>0.3 ± 0.2</td>
<td>2.4 ± 1.2</td>
<td>696 ± 308*</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> &amp; rolipram (5 × 10⁻⁷ M)</td>
<td>28.7 ± 18.5</td>
<td>107.3 ± 28.6</td>
<td>0.5 ± 0.3</td>
<td>2.0 ± 1.2</td>
<td>1522 ± 448</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> &amp; rolipram (10⁻⁷ M)</td>
<td>34.8 ± 14.8</td>
<td>154.6 ± 36.1</td>
<td>6.3 ± 6.2</td>
<td>1.7 ± 1.1</td>
<td>3800 ± 1006</td>
</tr>
</tbody>
</table>

The results are expressed as the density of bacteria adhering to a mucosal feature, calculated as described in the text. The mean total number of bacteria adhering to an organ culture is also given.

*P ≤ .04 vs. *P. aeruginosa* alone.

**TABLE 4**

<table>
<thead>
<tr>
<th>Method</th>
<th>Mucus</th>
<th>Damaged Mucosa</th>
<th>Ciliated Epithelium</th>
<th>Unciliated Epithelium</th>
<th>Total No. of Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> alone</td>
<td>11.7 ± 4.6</td>
<td>55.0 ± 13.9</td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.6</td>
<td>1164 ± 370</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> &amp; rolipram</td>
<td>3.4 ± 1.5</td>
<td>45.0 ± 14.9</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td>117 ± 46*</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> &amp; salmeterol</td>
<td>12.4 ± 6.8</td>
<td>39.2 ± 13.2</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>319 ± 103*</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> &amp; rolipram &amp; salmeterol</td>
<td>16.8 ± 8.4</td>
<td>62.0 ± 6.7</td>
<td>0.2 ± 0.08</td>
<td>1.2 ± 0.6</td>
<td>566 ± 357*</td>
</tr>
</tbody>
</table>

The results are expressed as the density of bacteria adhering to a mucosal feature, calculated as described in the text. The mean total number of bacteria adhering to an organ culture is also given.

*P ≤ .04 vs. *P. aeruginosa* alone.

**Fig. 2.** Effect of rolipram and salmeterol on *P. aeruginosa* growth in vitro (n = 3). ■ = *P. aeruginosa* alone; ▲ = *P. aeruginosa* and salmeterol (4 × 10⁻⁷ M); ▼ = *P. aeruginosa* and rolipram (1 × 10⁻⁶ M); ♦ = *P. aeruginosa* and rolipram and salmeterol (same concentrations).

**Fig. 3.** Effect of rolipram (1 × 10⁻⁶ M) and salmeterol (4 × 10⁻⁷ M) on *P. aeruginosa* culture filtrate-induced fall in intracellular cAMP levels (n = 6). Results are expressed as the mean ± S.E.M. *P ≤ .01 vs. *P. aeruginosa*. †P ≤ .01 vs. *P. aeruginosa* and salmeterol. ‡P ≤ .01 vs. *P. aeruginosa* and rolipram and salmeterol. P.A. = *Pseudomonas aeruginosa*, rol. = rolipram and salm. = salmeterol.
at inhibiting aerosol histamine or leukotriene D4-induced bronchospasm. Investigators have also focused on the anti-inflammatory action of drugs that inhibit PDE and so elevate cAMP. For example, iloprost, a stable prostacyclin analog, inhibits neutrophil-mediated lung injury in the rat (Riva et al., 1990) and prevents ultrastructural damage to hamster hepatocytes treated with paracetamol (Nasser-Sina et al., 1992). There is some evidence that cAMP might exert its cytoprotective effect via a calcium-dependent mechanism. Elevating intracellular cAMP increased the rate of extrusion of Ca\(^{2+}\) across the plasma membrane of human platelets via the Ca-ATPase pump (Johansson et al., 1992). Murata and colleagues (1994) demonstrated that superoxide-induced bleb formation on cultured rat hepatocytes was mediated via Ca\(^{2+}\), and Bickler and Hansen (1994) showed that membrane damage as judged by leakage of lactate dehydrogenase occurred coincidentally with calcium influx and ATP loss in rat cerebrocortical brain slices.

In our previous study of P. aeruginosa infection of the respiratory mucosa, salmeterol, a long-acting beta-2 agonist reduced epithelial damage caused by bacterial infection and also by the P. aeruginosa toxins pyocyanin and elastase. The mechanism of this protection was not elucidated, but it was inhibited by propranolol, which indicates that it was beta receptor-mediated (Dowling et al., 1997). In the present study, the effect of salmeterol in reducing the epithelial damage and loss of ciliated cells caused by P. aeruginosa infection was confirmed, and we demonstrated that rolipram protected the respiratory epithelium in a concentration-dependent manner. However, rolipram (1 \(\times\) 10\(^{-6}\) M) was significantly (P \(<\) .03) more effective than salmeterol in protecting the epithelium against P. aeruginosa-induced mucosal damage, but the two agents had similar effects on loss of ciliated cells. P. aeruginosa culture filtrate, like the bacterial toxin pyocyanin in our previous study (Kanthakumar et al., 1993), reduced the cAMP levels in epithelial cells. Rolipram preserved cAMP levels in the cells more than salmeterol, which suggests that this is the reason for its greater protective effect.

Preincubation of the tissue with rolipram (1 \(\times\) 10\(^{-6}\) M), salmeterol (4 \(\times\) 10\(^{-7}\) M) or both agents together (same concentrations) significantly (P \(<\) .04) reduced the total number of bacteria adhering to the respiratory mucosa without altering bacterial tropism for each mucosal feature (tables 3 and 4). This effect was probably due to a reduction in the amount of damage caused by P. aeruginosa to which the bacteria preferentially adhered (tables 1 and 2). In separate experiments we showed that rolipram, salmeterol or both agents together did not significantly affect P. aeruginosa growth in vitro (fig. 2). Damaged cells may release nutrients that stimulate bacterial growth, so reducing damage may also limit bacterial numbers in this way.

Suttorp and colleagues (1993) showed that rolipram (1 \(\times\) 10\(^{-6}\) M) blocked H\(_2\)O\(_2\)-induced endothelial permeability when combined with PGE\(_2\), a receptor-operated adenylate cyclase activator. A possible mechanism of action involves elevation of intracellular cAMP accompanied by protein kinase A activation, which might then decrease intracellular free Ca\(^{2+}\). A reduction in the Ca\(^{2+}\) signal reduces myosin light-chain kinase activity, leading to endothelial cell relaxation and closure of tight junctions (Rasmussen et al., 1990). Closure of tight junctions preserves the integrity of the endothelium and maintains the permeability barrier, thus preventing the influx of inflammatory cells. This mechanism may also operate for epithelial tight junctions and thus maintain epithelial integrity, which in turn may protect against epithelial damage. Rolipram has other anti-inflammatory actions. It significantly increased cAMP levels in BAL leukocytes, and it decreased BAL IL-8, TNF, eosinophils and neutrophils in response to allergen challenge in monkeys (Turner et al., 1994), as well as lipopolysaccharide-induced TNF synthesis by peripheral blood monocytes (Seldon et al., 1994). Because lipopolysaccharide induces cytokine production by epithelial cells (Khair et al., 1994), inhibition of their production by rolipram may be involved in the cytoprotective effect.

Tomlinson and colleagues (1995) used human airway smooth muscle cells to show that a combination of a nonspecific PDE inhibitor, IBMX, with the beta-2 agonist salbutamol produced an additive inhibitory effect on thrombin-induced mitogenesis. In the present study, we found that the combination of rolipram and salmeterol did not produce an additive or synergistic effect with respect to intracellular cAMP levels in epithelial cells exposed to P. aeruginosa culture filtrate, nor did it confer protection against P. aeruginosa-induced epithelial damage. In fact, surprisingly, rolipram and salmeterol together were significantly (P \(<\) .01) less effective than rolipram alone with respect to intracellular cAMP levels, and they seemed to be less effective than rolipram alone with respect to protection against P. aeruginosa-induced epithelial damage, although this difference was not significant. Torphy and colleagues (1992) showed that combined treatment of the human monocyte cell line U937 with the beta-2 agonist salbutamol (1 \(\mu\)M) and rolipram (30 \(\mu\)M) for 4 hr resulted in a 2- to 3-fold increase in PDE type IV activity. In the same system, rolipram alone was without effect and salbutamol alone had half the effect of both agents together with respect to PDE IV activity. Salmeterol is only a partial agonist with respect to cAMP, which could explain why rolipram increased cAMP levels more effectively than salmeterol in our study, and the results of Torphy and colleagues (1992) might explain why both agents together did not produce an additive or synergistic effect, because the up-regulated PDE activity might reduce intracellular cAMP levels.

In summary, our results show that two agents that elevate intracellular cAMP by different mechanisms protect the epithelium against damage caused by bacterial infection. Rolipram is more effective in achieving this than salmeterol. This may be because salmeterol is a partial agonist with respect to increasing intracellular cAMP levels. The two agents may interact together in some way such that their effect is not additive.

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References


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