Phosphodiesterase IV Inhibitors as Therapy for Eosinophil-induced Lung Injury in Asthma

Theodore J. Torphy, Mary S. Barnette, Douglas W.P. Hay, and David C. Underwood

Department of Inflammation and Respiratory Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania

Asthma is a complex, multifactorial disease that is underpinned by airway inflammation. A variety of cytotoxic substances are released into the airway from infiltrating inflammatory cells, especially the eosinophil. These cytotoxic substances, including reactive oxygen metabolites, produce damage to the airway epithelium, a histologic feature of chronic asthma. Damage to the airway epithelium, in turn, is thought to be a major factor responsible for the development of airway hyperreactivity, a hallmark of asthma. One notable molecular target for novel antiasthmatic drugs is the cyclic AMP-specific phosphodiesterase (PDE) or PDE IV. This isozyme is the predominant form of cyclic nucleotide PDE activity in inflammatory cells. Thus, in view of the putative role of cyclic AMP as an inhibitory second messenger in these cells, PDE IV inhibitors have been shown to suppress inflammatory cell activity. The purpose of the present experiments was to examine the effect of the PDE IV inhibitor, Rolipram, on three key functions of the guinea pig eosinophil: a) superoxide anion (O2-) production, b) adhesion to human umbilical vein endothelial cells (HUVECs), and c) infiltration into the airway. Rolipram-elevated eosinophil cyclic AMP content (EC50 = 1.7 µM) and inhibited fMLP-induced O2 production in a concentration-dependent manner (IC50 = 0.3 µM). In contrast, neither sisaguanodan, a PDE III inhibitor, nor zaprinast, a PDE V inhibitor, had an appreciable effect. Rolipram (30 µM) also reduced by 25 to 40% the adhesion of eosinophils to HUVECs stimulated with phorbol myristate acetate or tumor necrosis factor-α, particularly under conditions in which both cell types were simultaneously exposed to the PDE IV inhibitor. Again, sisaguanodan and zaprinast had little or no effect. Finally, pretreatment of conscious guinea pigs with Rolipram (1–10 mg/kg, intragastrically) produced a dose-dependent inhibition of antigen-induced eosinophil infiltration into the airway. Thus, by virtue of their ability to modify eosinophil function at several levels, PDE IV inhibitors may reduce epithelial cell damage associated with asthma. — Environ Health Perspect 102(Suppl 10):79–84 (1994)

Key words: asthma, eosinophil, airway hyperreactivity, epithelium, epithelial injury, superoxide, cyclic AMP, phosphodiesterase, phosphodiesterase inhibitors

Introduction

Asthma is a complex, multifactorial inflammatory disease of the airways (1,2). Both acute and chronic inflammatory processes lead to the two hallmarks of asthma, airway obstruction and airway hyperreactivity (2,3). Various mediators released from a number of inflammatory cells produce bronchoconstriction, pulmonary edema, and mucus secretion (3), three acute and reversible changes that result in airway obstruction. As chronic inflammatory processes proceed unchecked, more insidious and perhaps irreversible changes in airway architecture develop (4,5). Chief among these changes is loss of the airway epithelium (5). It is believed that the loss of the airway epithelium leads to airway hyperreactivity by exposing subepithelial sensory nerve endings to the external environment (6,7). Consequently, noxious environmental stimuli more easily activate these nerve endings to cause antidiromic release of bronchoconstricting neuropeptides (e.g., substance P) from C-fibers via an axon reflex (6,7).

The inflammatory cell primarily responsible for damaging the airway epithelium is the eosinophil (Figure 1). Eosinophils are recruited into asthmatic airways and activated by a variety of lipid mediators (e.g., leukotriene B4, platelet activating factor) and cytokines (e.g., tumor necrosis factor-α, interleukin-5) (3,8). Activated eosinophils release both proinflammatory and cytotoxic substances (3,8,9). In particular, these cells release cationic proteins (e.g., major basic protein, eosinophil cationic protein) and reactive oxygen metabolites (e.g., superoxide, singlet oxygen) (8–10). Hypothetically, these substances act in concert to destroy epithelial cell membranes. Indeed, the proposed role of eosinophil-derived reactive oxygen intermediates in producing epithelial damage has been supported by several in vitro and in vivo studies in both animals and humans (10–14). Recently, cyclic nucleotide phosphodiesterases (PDEs), a family of enzymes that metabolize cyclic AMP and cyclic GMP, have received considerable attention as molecular targets for novel antiinflammatory and antiasthmatic drugs (14,15). This interest has been fueled by the recognition that, in general, cyclic AMP suppresses the activity of immune and inflammatory cells (15,16). Thus, by virtue of their ability to elevate cyclic AMP content, PDE inhibitors possess antiinflammatory activity. Of particular interest as a drug target is the cyclic AMP-specific PDE, also known as PDE IV (15,16). This isozyme is the predominant PDE present in most inflammatory cells (15,16), including the eosinophil (17,18). Consequently, PDE IV inhibitors suppress the activity of eosinophils, as evidenced by their ability to reduce the generation of superoxide anion and H2O2 (17,18). In addition to the direct effect of PDE IV inhibitors on eosinophils, these compounds may suppress eosinophil function indirectly by producing a general reduction in the formation of lipid mediators and cytokines (15,16), thus inhibiting eosinophil migration and activation. Taken collectively, this information suggests that PDE IV inhibitors may be beneficial in...
reducing eosinophil-induced lung injury associated with chronic asthma.

The present studies were conducted to define the effects of isozyme-selective PDE inhibitors on eosinophil function in vitro and in vivo. Specifically, we examined the effects of rolipram, a PDE IV inhibitor (19), siguazodon, an inhibitor of the cyclic GMP-inhibited PDE (PDE III) (20), and zaprinast, an inhibitor of the cyclic GMP-specific PDE (PDE V) (21) on the following eosinophil functions: a) superoxide generation, b) endothelial cell adhesion, and c) infiltration into the airways.

Methods

Eosinophil Isolation and Purification

Peritoneal eosinophils were elicited by a modification of the procedure described by Gleich and Loegering (22). Male guinea pigs (Hartley, Hazleton Labs, Denver, PA) were injected with 1 ml of sterile horse serum weekly for 4 to 6 weeks prior to use. Animals were anesthetized with a mixture of 88 mg/ml ketamine and 12 mg/ml of xylazine (0.4 ml/kg) 24 hr after an injection of horse serum. After the induction of anesthesia, the peritoneal cavity was lavaged with 50 ml of warm sterile saline (0.9%) using a 14-gauge catheter, and the fluid collected in 50-ml plastic conical centrifuge tubes. The guinea pigs were allowed to recover from the anesthesia and could be used again after a 2-week rest period. No difference in either the recovery of cells or in the responsiveness of these eosinophils was noted if the animals were lavaged repeatedly.

To isolate eosinophils, the lavage fluid was centrifuged (400g, 10 min), the resulting pellet was resuspended in 35 ml of phosphate-buffered saline (PBS) and then layered with 10 ml of isotonic Percoll (1.075 g/ml). This suspension was centrifuged for 30 min at 300g. The pellet, containing mainly eosinophils and erythrocytes, was washed in PBS. The erythrocytes were lysed with distilled water (9 ml) and isotonicity was then restored by the addition of 10 \times PBS (1 ml). Eosinophils were resuspended in RPMI 1640 medium with 20% fetal bovine serum and incubated overnight at 37°C in a humidified 5% CO\textsubscript{2} incubator. The next day cells were washed and resuspended in PBS for determination of cell viability and purity.

Superoxide Anion Production

Superoxide anion (O\textsuperscript{2-}) production was determined by a modification of the microassay developed by Pick and Mizel (23). Purified eosinophils (viability >95% and purity >90%) were resuspended in Earle's balanced salt solution (EBS) with 20 mM HEPES buffer, pH 7.4, and 0.1% gelatin at a concentration of 1 to 2 \times 10\textsuperscript{6} cells/ml. Eosinophils (10\textsuperscript{6} cells per well) were added to a 96-well plate and incubated for 1 hr at 37°C. Eosinophils were pretreated with various concentrations of PDE inhibitors for 10 min prior to the start of the reaction. The reaction was initiated by the addition of FMLP (30 nM) and cytocrome c (160 \mu M) in the absence or presence of 60 U of superoxide dismutase. Cytochrome c reduction was monitored on a Dynatech (Chantilly, VA) MR 7000 plate reader at 550 nm with a 630-nm reference at several time points after the addition of FMLP. The production of O\textsuperscript{2-} was determined as the difference in absorbance between wells in the absence and presence of superoxide dismutase. Results are expressed as a percent of the control rate of cytochrome c reduction using the extinction coefficient of 21 \times 10\textsuperscript{2} M\textsuperscript{-1}cm\textsuperscript{-1}.

The maximum inhibition of O\textsuperscript{2-} generation produced by PDE IV inhibitors was 60%, the potencies of R- and S-rolipram were determined by the concentration that produced 30% inhibition (IC\textsubscript{30}) of the FMLP-stimulated rate. These values were calculated by linear interpolation using the mean response obtained from three to four experiments.

Measurement of Cyclic AMP Accumulation in Guinea Pig Eosinophils

Cultured guinea pig eosinophils were resuspended in EBS with 20 mM HEPES, pH 7.4, + 0.1% gelatin at a cell concentration of 3 \times 10\textsuperscript{6} cells/ml. Aliquots (100 \mu l) of cells were incubated with various concentrations of PDE inhibitors for 5 min at 37°C prior to the start of the reaction. The reaction was initiated by the addition of PGE\textsubscript{2} (10 \mu M) and continued for an additional 5 min. Cyclic AMP content was measured by RIA using commercially available kits (New England Nuclear, Boston, MA). Data were expressed as the increase in cAMP content/10\textsuperscript{6} cells over that seen with PGE\textsubscript{2} alone.

Eosinophil Adhesion

The experimental procedures employed in these studies were based on the method described by Schleimer and co-workers (24). Human umbilical vein endothelial cells (HUVECs), obtained from Cell Systems (Seattle, WA), were grown to confluence in Cell Systems (CS) complete medium on gelatin-coated 24-well plates. All experiments were conducted using cells passed four times. Eosinophils were isolated from guinea pigs by peritoneal lavage as outlined above. Eosinophils were labeled in a volume of 0.5 ml PAG buffer (Pipes, 25 mM; NaCl, 110 mM; KCl, 5 mM; human serum albumin, 0.003%; and glucose, 0.1%, pH 7.4) with 0.4 mM Cr sodium \textsuperscript{51}Cr chromate by incubation at 37°C for 60 min. The cells were washed four times in a large volume of PAG buffer and finally resuspended in PAGCM (PAG buffer plus CaCl\textsubscript{2}, 1 mM and MgCl\textsubscript{2}, 1 mM) at 1 \times 10\textsuperscript{6} cells/ml.

Phorbol myristate acetate (PMA; 0.1 \mu M) or tumor necrosis factor-\alpha (TNF\textalpha, 1000 U/ml) was added to the HUVECs while in CS medium and incubated in a CO\textsubscript{2} incubator at 37°C for 4 hr. After the 4-hr incubation, the medium containing the stimulant was removed completely and the cells were rinsed twice with warm PAGCM buffer. A total of 0.5 ml of warm PAGCM buffer containing 0.1 \times 10\textsuperscript{6}-labeled eosinophils was added to each well. The HUVECs and eosinophils were incubated at 37°C in a CO\textsubscript{2} incubator for 30 min and then each well was washed with PAGCM buffer to remove the unbound eosinophils. One-half milliliter 1 M NaOH..
was added to each well to dissolve the cells. The radioactivity present in the dissolved cells was measured using a gamma counter.

Three separate incubation conditions with the PDE inhibitors were used in the adhesion experiments:
- The PDE inhibitor was added to the HUVECs at the same time as PMA or TNF-α and allowed to incubate with the TNF-α or PMA for 4 hr. The PDE inhibitor was then washed out of the HUVECs and the adhesion assay was performed as outlined above.
- The PDE inhibitor was added to the labeled eosinophils and incubated for 30 min at 37°C. The cells were washed twice with buffer, added to the stimulated HUVECs, and the adhesion assay was performed as outlined above.
- The PDE inhibitor was added to the TNF-α or PMA-stimulated HUVECs just before the labeled eosinophils were added and the adhesion assay was performed as outlined above, with the PDE inhibitor present during the entire time.

### Phosphodiesterase Assay

The ability of compounds to inhibit human recombinant PDE IV activity was assessed as described previously (25).

### Antigen-induced Bronchoconstriction and Eosinophil Infiltration

The activity of R-rolipram was examined in a model of the early-phase/late-phase response to antigen as described previously (26). Male Hartley guinea pigs, sensitized to ovalbumin (OA), were pretreated with R-rolipram (1, 3, or 10 mg/kg, ig) or vehicle (polyethylene glycol) 1 hr before antigen challenge and chlorpheniramine (0.1 mg/kg, sc) 15 min before antigen challenge. The animals were placed into a two-chamber whole-body plethysmograph connected to a respiratory analyzer to determine specific airway conductance (sGaw) by a previously described method (26). An aerosol of 1% solution of OA was delivered for 10 sec, and pulmonary function was monitored for 10 min. Results were calculated as percent change in sGaw from baseline readings taken just prior to antigen challenge. Twenty-four hours after antigen challenge, bronchoalveolar lavage was carried out on the animals to document inflammatory cell (predominantly eosinophils) infiltration into the lungs (26).

### Results

#### Effects on Eosinophil Activation

Treatment of cultured eosinophils with either R-rolipram (10 μM) or the nonselective PDE inhibitor, 3-isobutyl-1-methylxanthine (100 μM), increased cyclic AMP content and suppressed fMLP-induced O₂⁻ production (Table 1). In contrast, the PDE IV inhibitor, siguazodan (100 μM), or the PDE V inhibitor, zaprinast (10 μM; not shown), had little or no effect on either parameter. The effects of rolipram were concentration dependent and R-rolipram was 3-fold more potent than S-rolipram as a PDE IV inhibitor (IC₅₀ = 0.31 ± 0.09 vs 1.10 ± 0.02 μM; n = 4) and 10-fold more potent as an inhibitor of O₂⁻ production (IC₅₀ = 0.3 vs 2 μM; n = 4) or as a stimulator of cyclic AMP accumulation (EC₅₀ = 2 μM vs 20 μM; n = 4).

To determine if other PDE IV inhibitors would inhibit eosinophil activation, 19 structurally diverse PDE IV inhibitors were evaluated for their ability to inhibit recombinant human PDE IV and suppress fMLP-induced O₂⁻ production. A strong rank-order correlation was demonstrated between the potencies of compounds as PDE IV inhibitors and their potencies as inhibitors of eosinophil activation (Spearman’s Rho = 0.85, p < 0.001). Similarly, a strong correlation existed between the absolute potencies of these compounds as inhibitors of PDE IV versus their potencies against eosinophil activation (r² = 0.68, p < 0.001).

#### Effects on Eosinophil Adhesion

Both PMA (0.1 μM) and TNF-α (1000 U/ml) markedly stimulated adhesion of guinea pig eosinophils to HUVECs. R-rolipram (10 or 30 μM) significantly inhibited the PMA-induced increase in adhesion only when both the eosinophils and HUVECs were exposed to the inhibitor (Figure 2). Siguazodan (30 μM), a selective PDE III inhibitor, had no effect on guinea pig eosinophil adhesion to HUVECs in any of the three incubation conditions (Figure 2). Similarly, zaprinast (30 μM), a selective PDE V inhibitor, was without effect on PMA-induced stimulation of adhesion (data not shown).

R-rolipram (30 μM) significantly inhibited TNF-α-stimulated adhesion of guinea-pig eosinophils to HUVECs in all three of the pretreatment conditions (Figure 3). The

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### Table 1. Effect of R-rolipram, siguazodan, and 3-isobutyl-1-methylxanthine on cyclic AMP accumulation and O₂⁻ generation in guinea pig eosinophils.

| Inhibitor | Cyclic AMP accumulation, a | O₂⁻ production, c |
|-----------|-----------------------------|-------------------|
| None      | 0.48 ± 0.05                 | —                 |
| IBMX, 100 μM | 2.65 ± 0.25*              | 54.8 ± 6.3*     |
| R-rolipram, 10 μM | 5.76 ± 1.16*              | 58.4 ± 18.1*    |
| Siguazodan, 100 μM | 0.66 ± 0.04                | 1.4 ± 4.9       |

IBMX, 3-isobutyl-1-methylxanthine. All values represent the mean ± SE. *Elevation of cyclic AMP content in the presence of 10 μM PGE₂ (n = 4). **Inhibition of fMLP-induced O₂⁻ production (n = 3-4) a p < 0.05.

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### Figure 2. Effect of R-rolipram (3, 10, and 30 μM) or siguazodan (30 μM) on PMA (0.1 μM)-stimulated adhesion of guinea pig eosinophils to human umbilical vein endothelial cells (HUVECs). Three different protocols were employed: a) exposure of HUVECs only with the PDE inhibitor for 4 hr followed by washout (open bars); b) exposure of eosinophils only with the PDE inhibitor for 30 min followed by washout (hatched bars); c) exposure of HUVECs and eosinophils with the PDE inhibitor throughout the adhesion assay (solid bars). Data represent the mean ± SE of six experiments. *p < 0.05.

### Figure 3. Effect of R-rolipram (3, 10, and 30 μM) or siguazodan (30 μM) on TNF-α (1000 U/ml)-stimulated adhesion of guinea pig eosinophils to human umbilical vein endothelial cells (HUVECs). Three different protocols were employed: a) exposure of HUVECs only with the PDE inhibitor for 4 hr followed by washout (open bars); b) exposure of eosinophils only with the PDE inhibitor for 30 min followed by washout (hatched bars); c) exposure of HUVECs and eosinophils with the PDE inhibitor throughout the adhesion assay (solid bars). Data represent the mean ± SE of six experiments. *p < 0.05.
degree of inhibition using the three protocols ranged from 25 to 40%, with cotreatment of eosinophils and HUVECs giving the greatest inhibitory effect. Sigaazodan (30 µM) also inhibited the adhesion of guinea pig eosinophils to HUVECs, but only when the cotreatment protocol was used. Zaprinast (30 µM) was without effect on TNFα-induced stimulation of adhesion using any of the three treatment protocols (data not shown).

**Effects on Antigen-induced Bronchoconstriction and Eosinophil Infiltration**

In vehicle-treated guinea pigs, aerosol OA produced a maximal decrease of 64% of baseline sGaw (Figure 4, top panel). Intragastric administration of R-rolipram (1, 3, or 10 mg/kg) 1 hr before antigen challenge inhibited the bronchospasm in a dose-dependent manner. In the same animals, the OA challenge markedly increased total leukocyte number, particularly eosinophils, as assessed by 24-hr bronchoalveolar lavage (Figure 4, bottom panel). Normal cell numbers recovered in unchallenged animals averaged 8.0 ± 1.0 × 10^5, of which 6 to 10% were eosinophils (not shown). Similar to its effect on antigen-induced bronchoconstriction, R-rolipram substantially reduced eosinophil influx into the lung in a dose-dependent manner. R-rolipram also produced a dose-dependent inhibition (29–48%) of total leukocyte infiltration.

**Discussion**

The role of eosinophil-derived reactive oxygen metabolites in producing damage to the airway epithelium has been recognized as a major factor in the pathophysiology of asthma (8–11,14). Consequently, drugs that modify eosinophil production, trafficking, or activation should be of use in the treatment of this disease. Isozyme-selective PDE IV inhibitors, typified by rolipram, represent one such class of drugs (15,16). These agents inhibit the activation of a variety of inflammatory cells, including the eosinophil (15–18). The present studies were conducted to explore the potential eosinophil-suppressant effects of PDE IV inhibitors. Specifically, we determined the effect of rolipram on the following eosinophil functions: a) FMLP-induced O2− generation, b) PMA- and TNFα-induced endothelial cell adhesion, and c) antigen-stimulated migration into the airway.

Production of O2− from FMLP-stimulated guinea pig eosinophils was inhibited strongly by rolipram and 3-isobutyl-1-methylxanthine, a nonselective PDE inhibitor, but not by sigaazodan or zaprinast, selective inhibitors of PDE III and PDE V, respectively. Several lines of evidence suggest that the inhibitory effect of rolipram was due to inhibition of PDE IV. First, the effect of rolipram on O2− production was accompanied by an increase in cyclic AMP accumulation. Second, similar to the enantioselectivity of rolipram against the catalytic activity of human recombinant PDE IV, R-rolipram was 10-fold more potent than S-rolipram with regard to both inhibition of O2− production and stimulation of cyclic AMP accumulation in intact eosinophils. Third, an excellent correlation was demonstrated between the potencies of 19 compounds for inhibition of human recombinant PDE IV and their potencies as inhibitors of FMLP-induced O2− production. These results support and extend previous reports indicating that PDE IV inhibitors reduce basal and opsonized zymosan-induced H2O2 and O2− generation from guinea pig eosinophils (17,18). In addition to its regulatory role in eosinophil function, cyclic AMP also alters endothelial cell function. Specifically, elevation of cyclic AMP content inhibits TNFα-induced expression of the adhesion molecules, endothelial leukocyte adhesion molecule (ELAM or e-selectin), and vascular cell endothelial molecule-1 (VCAM-1), but not intercellular adhesion molecule-1 (ICAM-1) (27). Moreover, like the eosinophil, endothelial cells contain PDE IV as their predominant cyclic AMP metabolizing enzyme (28,29). Thus, we hypothesized that PDE IV inhibitors could interfere with the adhesion of eosinophils to endothelial cells by acting at either, or both, cell types. Indeed, the results indicate that rolipram, but not sigaazodan or zaprinast, attenuated both PMA- and TNFα-induced adhesion of guinea pig eosinophils to HUVECs. Interestingly, the inhibitory effect of rolipram depended on the stimulus used. In cells stimulated with PMA, rolipram was active only when both the eosinophils and HUVECs were exposed to the inhibitor. In contrast, eosinophil adhesion to TNFα-stimulated HUVECs was inhibited if either the eosinophils or the endothelial cells were exposed to rolipram individually, although an even greater effect was observed if both cells were incubated with the inhibitor. The maximum inhibitory effect of rolipram in these studies was moderate, ranging from 25 to 40%. It is possible, however, that more pronounced inhibitory effects would be produced under conditions whereby adenyl cyclase activity in endothelial cells and eosinophils is elevated, e.g., in vivo under the influence of circulating catecholamines or locally released prostanooids (30,31). Such a result would be predicted based upon the synergistic interactions between PDE inhibitors and adenylyl cyclase activators in these cells (18,29).

In addition to its effects in in vitro settings, rolipram altered eosinophil function in vivo. Specifically, rolipram substantially reduced the influx of eosinophils into the airway that occurred over a 24-hr time period after aerosol administration of antigen to sensitized guinea pigs. Although the precise mechanism by which rolipram exerts this effect was not determined, several proposals can be made based upon a variety of in vitro actions of PDE IV inhibitors. For example, PDE IV inhibitors suppress mast cell degranulation (32) and may thus reduce the generation of chemo tactic lipid mediators (e.g., leukotriene B4, platelet activating factor) in response to antigen challenge. PDE IV inhibitors also have been shown to reduce TNFα generation (33) and antigen-driven IL-5 production (34). TNFα is believed to be critical in the recruitment and activation of eosinophils (35), whereas IL-5 is a major
eosinophil growth and differentiation factor (36). Finally, as demonstrated in the present study, rolipram has a direct inhibitory effect on eosinophil adhesion to endothelial cells. Any of these effects, individually or collectively, could account for the ability of PDE IV inhibitors to prevent antigen-induced eosinophil influx into the airways.

In summary, eosinophils have a crucial role in mediating oxidant-induced lung injury in asthma. Thus, agents that suppress eosinophil function may have a major impact on the morphologic changes in the airway that occur as a consequence of long-standing asthma. Phosphodiesterase IV inhibitors represent one class of agents that modify eosinophil function at several levels, ranging from a direct inhibitory effect on eosinophil activation to a broad suppression of eosinophil trafficking into sites of inflammation. As such, these agents represent a unique and exciting approach to the therapy of asthma.

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