Airway reactivity in vivo and in vitro

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Bronchodilatory and anti-inflammatory properties of inhaled selective phosphodiesterase inhibitors in a guinea pig model of allergic asthma.

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SUMMARY

In a guinea pig model of allergic asthma, we investigated the effects of the selective phosphodiesterase (PDE) inhibitors rolipram (PDE4-selective), Org 9935 (PDE3-selective) and Org 20241 (dual PDE4/PDE3-selective), administered by aerosol inhalation in approximately equipotent bronchodilatory doses, on allergen-induced early (EAR) and late (LAR) asthmatic reactions, airway hyperreactivity (AHR) and airway inflammation.

Using ovalbumin (OA)-sensitized non-challenged animals, different nebulizer concentrations of each inhibitor were tested for their protective effects against histamine-induced bronchoconstriction. Inhalation of 2.5 mM rolipram, 100 mM Org 9935 and 10 and 100 mM Org 20241 provided a similar, 1.8-fold (P<0.01), 2.0-fold (P<0.05), and 1.8- and 1.9-fold (P< 0.05) protection, respectively. The duration of these bronchoprotective effects were different, the rate of decline being faster with rolipram and the lower Org 20241 concentration than with Org 9935 and the higher concentration of Org 20241.

All compounds strongly protected against the immediate allergen-induced bronchoconstriction and significantly (P<0.05) diminished the overall EAR from 0 to 6h following allergen-provocation. The severity of the LAR was also significantly inhibited by rolipram (P<0.05) and Org 9935 (P<0.05).

Allergen-induced AHR to inhaled histamine after the EAR, at 6h after OA-challenge, was strongly reduced by rolipram (P<0.05) and completely prevented by the two other PDE inhibitors; in addition, AHR after the LAR, at 24h, was abolished in all treatment groups.

Bronchoalveolar lavage performed at 24h after allergen challenge revealed no inhibition of eosinophil infiltration in the rolipram-treated animals whereas inhalation of Org 9935 and the higher - but not the lower - concentration of Org 20241 strongly reduced the influx of these cells. Eosinophil peroxidase activity in the lavage fluid tended to be diminished in all treatment groups but significance was not reached with the exception of the lower concentration of Org 20241. Infiltration of lymphocytes and macrophages was significantly inhibited by Org 9935 only (P<0.05 and P<0.01, respectively) whereas neutrophil influx was not significantly affected.

The results indicate that inhalation of PDE3-, PDE4- and dual PDE3/PDE4-selective inhibitors afford protection against acute histamine- and allergen-induced bronchoconstriction and prevent the development of airway hyperreactivity both after the early and late asthmatic reaction predominantly through inhibition of PDE4; in contrast, for significant reduction of eosinophil infiltration both PDE3 and PDE4 inhibition seems to be required.

INTRODUCTION

Theophylline has been used in the treatment of asthma for over 50 years, while about twenty years ago, it has been suggested that theophylline, as a methylxanthine, acts as a cyclic nucleotide phosphodiesterase inhibitor (see Banner and Page, 1995).
In the past years, evidence from biochemical, functional and molecular biological studies has revealed the existence of a large superfamily of cyclic nucleotide phosphodiesterase isoenzymes (PDE), which consists of 7 distinct families, (some of them having different isoforms), based on substrate specificity, sensitivity to selective inhibitors and the effect of allosteric modulators (for review see Beavo, 1995). Since the distribution of these subtypes is different for various tissues, subtype-selective PDE inhibitors would offer the possibility of selective regulation of tissue function, with lesser unwanted side-effects, as known for theophylline in the treatment of asthma (Nicholson et al., 1991). Therefore, isoenzyme-selective PDE inhibitors are being explored as a new and promising class of drugs with anti-inflammatory and bronchodilatory properties that may be used in the therapeutic intervention of asthma (Nicholson and Shahid, 1994; Banner and Page, 1995; Torphy, 1998).

In airway smooth muscle from animals and man the presence of at least five PDE subtypes (PDE 1, 2, 3, 4 and 5) has been demonstrated (Torry and Cieslinsky, 1989; Shahid et al., 1991; De Boer et al., 1992; Torphy et al., 1993). In human airways selective inhibitors of PDE 3 and PDE 4 are potent bronchodilators (De Boer et al., 1992), and in canine tracheal preparations Torphy et al. (1988; 1991) demonstrated a potentiation of the isoprenaline-induced relaxation and increase of cAMP by selective PDE3 and PDE4 inhibitors. Furthermore, in other species it has been found, both in vitro and in vivo, that respiratory smooth muscle tone is regulated by both PDE3 and PDE4 (Harris et al., 1989; Heaslip et al., 1991). In addition, PDE4 and to a lesser extent PDE3 seem to be the dominant isoenzymes responsible for the breakdown of cAMP in inflammatory cells (Banner and Page, 1995; Torphy, 1998).

Thus, in eosinophils, which are considered important in the pathology of allergic asthma, it has been found that selective inhibition of PDE4, the predominant isoform, attenuates superoxide anion generation, opsonised zymosan-induced respiratory burst, and LTB4 generation (Dent et al., 1991, Souness et al., 1991; Banner et al., 1996). In addition, rolipram (a PDE4 selective inhibitor) decreases eotaxin-induced upregulation of CD11b and transendothelial chemotaxis (Santamaria et al., 1997). In a guinea pig eye model of tissue eosinophilia, rolipram reduces both histamine- and leukotriene-evoked eosinophil infiltration in the conjunctiva (Newsholme and Schwartz, 1993), and in sensitised Brown Norway rats rolipram and Org 20241 (a dual type 3- and 4-selective PDE inhibitor) abolished allergen-induced eosinophilia and neutrophilia (Elwood et al., 1995). Furthermore, it has been demonstrated that PDE 4-selective inhibitors suppress TNF-α production from endotoxin stimulated human monocytes (Molnar-Kimber et al., 1993; Semmler et al., 1993). In addition, antigen-specific Th1 and Th2 lymphocyte cytokine gene expression and release is being regulated differentially by PDE4 inhibition (Essayan et al., 1997).

Some reports have indicated that a combined inhibition of PDE3 and PDE4 may be more effective than single inhibition of either isoenzyme. Thus, in T-lymphocytes combined inhibition of PDE3 and PDE4 produces a synergistic antiblastogenic effect and only the combined inhibition of both isoenzymes completely suppresses T-lymphocyte proliferation (Robicsek et al., 1991; Schudt et al., 1995). Also, in human alveolar macrophages LPS-induced release of TNFα is only completely inhibited by a combination of rolipram and motapizone (PDE3-selective) (Schudt et al., 1995). Furthermore, in guinea pig tracheal
preparations only a combination of PDE3 and PDE4 inhibition causes complete relaxation of carbachol induced contractions (Harris et al., 1989). These findings indicate that mixed PDE3 and PDE4 selective inhibitors may have additional anti-inflammatory and bronchodilating potencies.

In a previous study, using a guinea pig model of allergic asthma (Santing et al., 1992; 1994a), inhibitory effects of low subbronchodilating, i.p. administered doses of the PDE inhibitors theophylline (non-selective), rolipram (PDE 4 selective) and Org 20241 (dual PDE3 and PDE4 selective) (Nicholson et al., 1995) were observed on the allergen-induced increase in bronchial reactivity both after the early (EAR) and after the late asthmatic reaction (LAR), at 6h and at 24h after allergen provocation, respectively, and on airway inflammation at 24 h after allergen challege (Santing et al., 1995); a PDE3 selective inhibitor was not used in that study, however. In the present study we now investigated the effects of rolipram, Org 9935 (PDE3 selective) and Org 20241, administered by aerosol inhalation in bronchodilating doses, on allergen-induced EAR and LAR, bronchial hyperreactivity after these reactions, and on influx and activation of inflammatory cells.

**METHODS**

*Animals*

Outbred specific pathogen free guinea pigs (Charles River SAVO, Kiszlegg, Germany), weighing 600 - 800 g, were used in this study. All animals were actively IgE-sensitised to ovalbumin at three weeks of age as described by Van Amsterdam et al. (1989). In short, 0.5 ml of an allergen solution containing 100 µg ml⁻¹ ovalbumin and 100 mg ml⁻¹ Al(OH)₃ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck.

For pleural pressure (Pₚₛₚ) measurements the animals were operated on in week 3 following sensitisation and used experimentally in weeks 4 to 8. After the operation the animals were housed in individual cages, in climate controlled animal quarters and given water and food *ad libitum*.

All protocols described in this study were approved by the University of Groningen Animal Health Committee.

*Measurement of airway function*

Airway function was assessed by on-line measurement of Pₚₛₚ under unrestrained conditions as described by Santing et al. (1992). Briefly, a small latex balloon (HSE, Freiburg, Germany), connected to a saline-filled canula, was surgically implanted inside the thoracic cavity. The canula was driven subcutaneously to and permanently attached in the neck of the animal. After connection via another saline-filled canula to a pressure transducer (TC-XX, Viggo-Spectramed B.V., Bilthoven, The Netherlands), Pₚₛₚ was measured, using an on-
line computer system. No postoperative inflammation was observed for at least 5 weeks after operation, and baseline $P_{pl}$ values remained stable during repeated measurements.

Using a combination of flow measurement with a pneumotachograph, implanted in the trachea, and pressure measurement with the pleural balloon, it was shown previously that changes in $P_{pl}$ are linearly related to changes in airway resistance and hence can be used as a sensitive index for histamine- and allergen-induced bronchoconstriction (Santing et al., 1992).

**Provocation techniques**

Ovalbumin and histamine provocations were performed by inhalation of aerosolised solutions. The provocations were performed in a specially designed animal cage, in which the guinea-pigs could move freely (Santing et al., 1992). The volume of the cage was 9 l, which ensured fast replacement of the air inside the cage with aerosol and vice versa. A DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA) driven by an airflow of 8 l min$^{-1}$ provided the aerosol required, with an output of 0.33 ml min$^{-1}$.

Habituation of the animals to the provocation conditions started two days before the experimental protocol. On the first day, the animals were placed inside the provocation cage unconnected to the transducers. Three consecutive challenges with saline solution were performed lasting three min each and separated by 10-min intervals. The next day, this protocol was repeated with the animals connected to the measuring system. On the first day of the experimental protocol, baseline histamine $PC_{100}$ was assessed, which was repeated on the second day.

Histamine provocations were performed starting with a concentration of 25 µg ml$^{-1}$ in saline, followed by increasing dosage steps of 25 µg ml$^{-1}$. The provocations by each concentration lasted 3 min and provocations were separated by 7-min intervals. The animals were challenged until the $P_{pl}$ increased by more than 100% for at least 3 consecutive min during the 10-min period. The provocation concentration causing a 100% increase in $P_{pl}$ ($PC_{100}$) was derived by linear interpolation.

Allergen provocations were performed by inhalation of increasing aerosol concentrations containing 1.0, 3.0, 5.0 and 7.0 mg ml$^{-1}$ ovalbumin in saline for 3 min, separated by 7-min intervals. Allergen inhalations were discontinued when an increase in $P_{pl}$ of more than 100% was observed. Using these conditions, no antihistaminic was needed to prevent the development of anaphylactic shock. All histamine and ovalbumin provocations were preceded by a period of at least 30 min for adaptation of the animals to the cage, followed by two consecutive inhalations with saline solution, lasting 3 min each and separated by a 7-min interval.

**Provocation protocols**

To determine the effectiveness against histamine-induced bronchoconstriction, thirty minutes after a control histamine $PC_{100}$-measurement the lowest concentration of the PDE inhibitor (rolipram: 25 µM; Org 9935: 1.0 mM; Org 20241: 1.0 mM) was nebulized for 15
minutes. After another 15 minutes a second histamine PC\textsubscript{100}-determination was performed, starting at 2 concentration steps below the initial PC\textsubscript{100}. Using 10-fold increased concentrations of the PDE inhibitor this procedure was two times repeated after 30 minutes. PC\textsubscript{100}-values were expressed as percent change from the control value in the same animal.

To determine the duration of the effectiveness against histamine-induced bronchoconstriction, 30 minutes after an initial histamine PC\textsubscript{100}-measurement a concentration of histamine was nebulized 10 \% above the PC\textsubscript{100}-value. The resulting increase in P\textsubscript{pl} was used as control value. After another 30 minutes the selected concentrations of the PDE inhibitors were nebulized for 15 minutes (rolipram: 2.5 mM; Org 9935: 100 mM; Org 20241: 10.0 mM and 100 mM). Subsequently, at 20, 40, 60, 90, 120, 150, 180, 240, 300, 360, 480, 600, 720 minutes after treatment, the PC\textsubscript{110} concentration of histamine was administered again and the resulting increase in P\textsubscript{pl} was expressed as percent inhibition of the control value.

The effectiveness of the PDE inhibitors against allergen-induced early and late phase reactions, bronchial hyperresponsiveness and airway inflammation was determined by administering an aerosol of the PDE inhibitors for 15 minutes, 1h prior to the second allergen provocation, which was identical to and separated by 7 days from the first, control, allergen provocation. After the allergen provocation the animals were removed from the provocation cage and placed in their individual larger home-cages of 2500 cm\textsuperscript{2}, were they could eat and drink ad libitum. To establish the change in airway reactivity at 6h (between the early and late asthmatic response; Santing \textit{et al.}, 1992,1994a) and at 24h after allergen provocation, the animals were placed in the provocation cage and the PC\textsubscript{100} value for histamine was re-assessed. During the transfer, the animals remained connected to the measurement system. Only animals that displayed a dual response to inhaled allergen (i.e. animals with both an EAR and a LAR) were used in this study protocol, since bronchial hyperreactivity is significantly more pronounced in these animals (Santing \textit{et al.}, 1994a).

\textit{Bronchoalveolar lavage (BAL) procedure}

At 24h after the second allergen provocation animals were anaesthetised with 20 mg/ml Brietal-sodium, 35 mg/kg ketamine hydrochloride and 6 mg/kg Rompun i.p. which ensured a fast, deep anaesthesia. The lungs were gently lavaged via a tracheal canula with 5 ml of sterile saline at 37 °C, followed by three subsequent aliquots of 8 ml saline. The recovered samples were placed on ice, and centrifuged at 200 g for 10 minutes at 4 °C. The supernatants of the first fractions were rapidly frozen for additional determination of eosinophil peroxidase (EPO) activity. Subsequently, the combined pellets were resuspended to a final volume of 1.0 ml in RPMI-1640 medium and total cell numbers were counted in a Bürker-Türk chamber. For cytological examination, cytospin-preparations were stained with May-Grünwald and Giemsa. A cell differentiation was performed by counting at least 400 cells in duplicate.
**Eosinophil peroxidase (EPO) assay**

The EPO activity in the supernatant of the first BAL fraction, as an indication of eosinophil activation, was analysed according to the kinetic assay described by White and colleagues (1991), which is based on the oxidation of o-phenylenediamine (OPD) by EPO in the presence of hydrogen peroxide (H$_2$O$_2$). Substrate was made by dissolving 0.018 % H$_2$O$_2$ and 16 mM OPD in 100 mM Tris-HCl buffer, pH 8.0, containing 0.1 % Triton X-100, immediately before use.

For the assay, 50 µl of supernatants were combined with 75 µl of substrate in a polystyrene 96-well microplate and placed into a thermoregulating microplate absorbance spectrophotometer (Thermomax, Molecular Devices, Menlo Park, Ca, USA) at 37°C. Absorbance at 490 nm was measured every 5 sec for 30 min; the velocity of the reaction (mOD/min.) was calculated by interpolation between successive 20 points (5 min) utilising customised software (Softmax v2.01, Molecular Devices). All samples were assayed in quadruplicate.

**Data analysis.**

Changes in the *in vivo* airway reactivity to histamine induced by allergen provocation were expressed as the ratio of histamine PC$_{100}$ values obtained 24h before and 6 and 24h after the allergen provocation, respectively (PC$_{100}$ ratio pre/post allergen challenge). The magnitude of the allergen-induced EAR and LAR was expressed as the area under the P$_{pt}$-time curve (AUC) from 0 to 6h and from 8 to 24h after allergen provocation respectively, as calculated by trapezoid integration (Santing *et al.*, 1994a).

The results are expressed as means ± s.e. mean. Statistical analysis was performed using the Student's t-test for paired or unpaired observations as appropriate. Differences were considered statistically significant at P<0.05.

**Preparation of the drug solutions**

For the preparation of Org 9935 and Org 20241 suspensions, micronised drugs were used. A 2.5 % soja-lecithin solution in saline, prepared 14h before use by gently stirring overnight, was used to avoid aggregation of the particles. Shortly before use the micronised drug (particle size approximately 75 µm in both cases) was added to the soja-lecithin solution, which was then placed in an ultrasonificator for 5 minutes. Rolipram was dissolved in saline, using the ultrasonificator if required. The soja-lecithin solution or saline were used as controls.
Chemicals

Histamine hydrochloride, ovalbumin (grade III), aluminum hydroxide, o-phenylenediamine dihydrochloride, and May-Grünwald and Giemsa stain were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Brietal-sodium (methohexital) was from Eli Lilly (Amsterdam, the Netherlands), ketamine hydrochloride from Parke-Davis (Barcelona, Spain), Rompun (2-(2.6-xylidino)-5.6-dihydro-4H-1.3-thiazine-hydrochloride, methylparaben) from Bayer (Leverkusen, Germany), and RPMI-1640 medium and Hanks balanced salt solution (HBSS) from Gibco Life Technologies (Praisley, Schotland). Rolipram was a gift from Schering, Berlin, Germany, Org 20241 (N-hydroxy-4-(3,4-dimethoxyphenyl)-thiazole-2-carboximidamide HCl), and Org 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thien-2-yl-5-methyl-3(2H)pyridazinone) were gifts from, Organon Laboratories (Newhouse, Lanarkshire, UK).

RESULTS

Effectiveness against histamine-induced bronchoconstriction

Inhalation of the vehicles and the PDE inhibitors at all concentrations used did not change basal pleural pressure (data not shown).

The effectiveness of the PDE inhibitors against histamine-induced airway obstruction in
sensitized guinea pigs as expressed as a percentage of the PC\textsubscript{100}-value determined before inhalation of the PDE inhibitor is depicted in Figure 1. For rolipram (PDE4-selective) only the highest concentration (2.5 mM) caused a significant inhibition of histamine-induced bronchoconstriction (1.8-fold increase of the control PC\textsubscript{100}, P<0.01). Time-matched, vehicle (saline) administrations were without significant effect.

The PDE3-selective inhibitor Org 9935 caused a 1.5-fold inhibition of histamine-induced airway obstruction at 10 mM (P<0.05) and a 2.0-fold inhibition at 100 mM (P<0.05). The same concentrations Org 20241 dual (PDE3/4-selective) induced a 1.8-fold (P<0.05) and a 1.9-fold (P<0.05) inhibition, respectively. Soja-lecithin administrations were without significant effect. Based on the above observations, active concentrations of 2.5 mM rolipram, 100 mM Org 9935 and 10 mM as well as 100 mM Org 20241 were chosen for further study.

**Duration of inhibition of histamine-induced bronchoconstriction**

The duration of the effect of the PDE inhibitors against histamine-induced bronchoconstriction was determined by monitoring the increase in P\textsubscript{pl} induced by a fixed dose of aerosolised histamine (PC\textsubscript{110}) at several time points after single administration of the PDE inhibitor (Figure 2).

The protective effect of 2.5 mM rolipram against histamine-induced bronchoconstriction
remained significant for 90 minutes; 180 minutes after rolipram administration the effect had vanished in all 6 animals. The duration of the inhibitory effect of 100 mM Org 9935 was 120 minutes; the time-response curve showed a more gradual decline than with rolipram. The lower concentration of Org 20241 (10 mM) gave a significant protective effect against histamine-induced bronchoconstriction that lasted for 90 minutes; histamine-induced bronchoconstructions returned to control levels in all 7 animals at 180 minutes after 10 mM Org 20241 administration. The higher concentration of Org 20241 (100 mM) caused a stronger inhibition of histamine-induced bronchoconstriction, which remained statistically significant during 180 minutes. Inhibition was still present in some animals at 480 minutes after inhalation of this concentration of Org 20241.

**Effectiveness against allergen-induced EAR, LAR and bronchial hyperreactivity**

The data of the allergen-induced early and late reactions (presented as AUC) before and after treatment with vehicle and the PDE inhibitors are presented in Table 1. The immediate (maximal) increase in $P_{pl}$ after allergen provocation, which is predominantly determined by mediators released after acute mast cell degranulation, is also presented in Table 1.

**Table 1:** Effects of phosphodiesterase inhibitors on allergen-induced early and late asthmatic reactions, and on the maximal height of the early reaction

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Early Asthmatic Reaction (EAR)</th>
<th>Late Asthmatic Reaction (LAR)</th>
<th>maximal height EAR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before treatment</td>
<td>after treatment</td>
<td>before treatment</td>
</tr>
<tr>
<td>Saline</td>
<td>3476 ± 1313</td>
<td>3182 ± 1212</td>
<td>9517 ± 1842</td>
</tr>
<tr>
<td>Rolipram (2.5 mM)</td>
<td>3460 ± 623</td>
<td>1178 ± 273 *</td>
<td>5251 ± 1252</td>
</tr>
<tr>
<td>Soja-lecithin</td>
<td>2808 ± 608</td>
<td>1610 ± 546</td>
<td>11831 ± 1966</td>
</tr>
<tr>
<td>Org 9935 (100 mM)</td>
<td>4402 ± 728</td>
<td>1271 ± 583 *</td>
<td>11036 ± 1218</td>
</tr>
<tr>
<td>Org 20241 (10 mM)</td>
<td>3918 ± 725</td>
<td>2281 ± 891</td>
<td>8206 ± 2634</td>
</tr>
<tr>
<td>Org 20241 (100 mM)</td>
<td>2656 ± 630</td>
<td>567 ± 491 *</td>
<td>11367 ± 3133</td>
</tr>
</tbody>
</table>

Inhalation of vehicle (saline or soja-lecithin), rolipram, Org 9935 and Org 20241 1h before the second allergen provocation. Data are presented as area under $P_{pl}$ time-response curve between 0 and 6h after allergen provocation for the EAR and between 8 and 24h after allergen provocation for the LAR. Maximal height of the EAR is expressed as % change of $P_{pl}$. Data represent mean values ± S.E.M. for 4 - 7 animals. Statistical analysis: Student’s t-test for paired observations (before/after treatment). *: P<0.05; **: P<0.01.

No significant effects were observed with the vehicle solutions - saline and 2.5% soja-lecithin in saline, respectively - on the EAR, LAR and the early peak increase in $P_{pl}$ during the EAR. However, inhalation of PDE inhibitors 1h prior to the second allergen provocation significantly inhibited the peak increase in $P_{pl}$ during the EAR. Moreover,
except for the lower concentration of Org 20241 (10 mM), the severity of the EAR, was also significantly inhibited by the PDE inhibitors. In addition, Org 9935 (100 mM) and rolipram (2.5 mM) significantly reduced the severity of the LAR, whereas both concentrations Org 20241 (10 mM and 100 mM) showed a tendency towards reduction of the LAR; this was also observed in the soja-lecithin control group, however.

The effect of the PDE inhibitors on the bronchial hyperreactivity measured at 6h (after the EAR) and 24h (after the LAR) after allergen provocation is presented in Figure 3. All PDE inhibitors significantly suppressed the allergen-induced increase in bronchial reactivity at 6h after allergen provocation; remarkably, in the animal groups treated with Org 9935 (100 mM) and Org 20241 (both concentrations) the development of the early bronchial hyperreactivity was completely prevented.

The development of bronchial hyperreactivity observed after the LAR was also prevented, both by rolipram, Org 9935 and by the higher concentration of Org 20241; the inhibitory effect of the lower Org 20241 concentration was nearly significant (P=0.06).

**Effects on allergen-induced inflammation**

The analysis of the cellular contents of the bronchoalveolar lavage obtained 24h after the second allergen provocation is depicted in Figure 4. Allergen-induced influx of eosinophils was not significantly affected by prior inhalation with rolipram but markedly suppressed by Org 9935 and the higher concentration of Org 20241. Neutrophil influx tended to be decreased by all PDE inhibitors (except the lower concentration of Org
Lymphocyte and macrophage numbers were only decreased significantly by Org 9935. The effect on the EPO activity measured in the BAL-fluid is shown in Figure 5. After inhalation of all PDE inhibitors a tendency towards decreased EPO-activity was observed; however, only with the lower Org 20241 concentration this reduction reached statistical significance.

**DISCUSSION**

In a previous study, using a guinea pig model of allergic asthma, the effects of subbronchodilatory doses of theophylline, rolipram and Org 20241, administered via the intraperitoneal route, on allergen-induced EAR, LAR, bronchial hyperreactivity, and airway inflammation were studied (Santing et al., 1995). Marked inhibition of the hyperreactivity to histamine, both after the EAR and LAR, were observed with all drugs whereas the severity of the EAR and the LAR were not affected; influx of both eosinophils and neutrophils was reduced in the theophylline treated animals whereas rolipram preferentially inhibited neutrophil influx and Org 20241 eosinophil infiltration (Santing et al., 1995)

The primary aim of the present study, using the same guinea pig model of allergic asthma, was to compare the effects of a PDE3-, a PDE4- and a dual PDE3/PDE4-inhibitor, when given in bronchodilating doses through the inhalational route. To select approximately equipotent nebulizer concentrations of the drugs, different concentrations of each PDE inhibitor were tested for their protective effects against histamine-induced bronchoconstriction in non-challenged animals; to this purpose sequential PC_{100} determinations for histamine were performed before and after inhalation of the drugs. The results showed that rolipram (PDE4 selective) at 2.5 mM, Org 9935 (PDE3 selective) at 100 mM and Org 20241 (dual PDE3/PDE4 selective) at 10 and 100 mM provided a similar 1.8-, 2.0-, and 1.8- and 1.9- fold protection, respectively. Although these concentrations seem to be high, it is more appropriate to calculate the total administered dose during the 15 min inhalation; for rolipram this amounted to 12.5 μmol (approximately 3.5 mg). Using intravenous administration, similar doses (3-10 mg/kg) of rolipram were required to reduce
PDE inhibition and allergic asthma

Histamine-induced bronchoconstriction in anaesthetized, ventilated guinea pigs (Underwood et al., 1993). Actually, in our set-up only a fraction of the total nebulized dose reaches the airways, since the PDE inhibitor is nebulized into a 9 liter chamber in which the animal could move freely.

Rolipram was found effective against histamine-induced bronchoconstriction at an about 40 times lower concentration than with Org 20241 and Org 9935. This is no surprise because the high potency of rolipram to relax airway smooth muscle is more related with the ‘high affinity rolipram binding PDE4’ (Harris et al., 1989; Souness et al., 1997). Remarkably, Org 20241, having a more shallow dose-effect relationship than Org 9935, was essentially equipotent to the PDE3 inhibitor, despite the fact that Org 9935 is at least 100-fold selective for inhibiting PDE3 compared to PDE4, whereas Org 20241 is a dual inhibitor with some selectivity for PDE4 (Nicholson et al., 1995). Indeed, the high potency and selectivity of Org 9935 for inhibiting the PDE3 isoenzyme was reflected in a biphasic relaxation curve of histamine-contracted guinea pig tracheal smooth muscle, in vitro the first phase being approximately one log-unit left from the Org 20241 curve (Nicholson et al., 1995). It might be concluded, therefore, that the protection against histamine-induced bronchoconstriction in vivo by the two PDE inhibitors is mainly due to inhibition of PDE4.

The duration of the protection by the selected concentrations of the PDE inhibitors was different, the rate of decline being faster with rolipram than with Org 9935; interestingly, the lower Org 20241 concentration showed a rolipram-like picture whereas the decline of the higher concentration of this compound had an Org 9935-like appearance. Though these differences would suggest that a longer duration of action in some way was associated with PDE3 inhibition, recent data in guinea pig airways by Spina et al. (1998) have shown that the duration of action of PDE inhibitors is independent of isoenzyme-selectivity.

As expected, all compounds strongly protected against the immediate allergen-induced bronchoconstriction and diminished the overall EAR from 0 to 6h following allergen provocation.

The LAR was also significantly inhibited by rolipram and Org 9935, whereas Org 20241 did not provide a significant protection. Although the inhibition of the EAR may partially be attributed to the direct bronchodilating effects of the PDE inhibitors, the inhibition of the LAR by Org 9935 and (particularly) rolipram indicates an anti-inflammatory effect. This is also clearly demonstrated by the prevention of the development of allergen-induced eosinophil peroxidase activity in bronchoalveolar lavage at 24h after the second allergen provocation. Control data are from sensitised, non-challenged animals. Results are presented as mOD/min from the first bronchoalveolar lavage supernatant. Data are presented as mean ± s.e.m. from 4-7 animals. Statistical analysis: unpaired Student’s t-test. *:P<0.05, compared to control; #:P<0.05 compared to soja-lecithin.

Figure 5 Eosinophil peroxidase activity in bronchoalveolar lavage at 24h after the second allergen provocation. Control data are from sensitised, non-challenged animals. Results are presented as mOD/min from the first bronchoalveolar lavage supernatant. Data are presented as mean ± s.e.m. from 4-7 animals. Statistical analysis: unpaired Student’s t-test. *:P<0.05, compared to control; #:P<0.05 compared to soja-lecithin.
bronchial hyperreactivity, both after the EAR (at 6h) and after the LAR (at 24h after allergen provocation) by all PDE inhibitors. It should be mentioned that at 6h, the direct bronchodilatory effects of rolipram, Org 9935 and the lower concentration of Org 20241 have completely disappeared already.

The inflammatory cell numbers measured after the LAR showed a differential picture, however. Eosinophil infiltration was not inhibited in the rolipram-treated animals whereas inhalation of Org 9935 and the higher - but not the lower - concentration of Org 20241 strongly reduced the influx of these cells. From these results the picture would emerge that inhibition of both PDE3 and PDE4 activity (by Org 9935 and the higher concentration of Org 20241) rather than of PDE4 alone, is involved in the reduction of eosinophil migration.

Interestingly, in our previous study in which subbronchodilatory doses were studied using i.p. administration, similar results were obtained: no significant inhibition by rolipram but a clear reduction of eosinophil influx by Org 20241 and theophylline. Underwood et al. (1994) also observed that ovalbumin-induced pulmonary eosinophil influx was attenuated most prominently by a mixed PDE3/4 inhibitor compared to selective PDE3 or PDE4 inhibitors. However, other studies have clearly demonstrated antigen-induced infiltration of eosinophils to be decreased by selective PDE4 inhibitors, including rolipram, RO 20-1724 and RP 73401 (Underwood et al., 1993; 1997; Raeburn et al., 1994; Ortiz et al, 1996; Danahay & Broadley, 1997). In these studies, the PDE4 inhibitors were however administered via the intraperitoneal, intragastric or intravenous route or by tracheal instillation whereas in the present study the drugs were administered through inhalation. The former modes of administration have in common that inflammatory cell trafficking is being influenced by the drugs from the vascular compartment whereas aerosol inhalation allows the drugs to approach airway target cells, including mast cells, from the airway lumen.

Direct inhibition of mast cell degranulation by the inhaled PDE inhibitors would reduce the generation of chemotactic mediators involved in eosinophil recruitment. Since both PDE3 and PDE4 are present in lung mast cells (Weston et al, 1997), dual inhibition of both isoenzymes could be more effective than PDE4 alone. As far as the infiltration of neutrophils, lymphocytes and macrophages is concerned, the only significant inhibition after aerosol administration was with Org 9935, again indicating some role of PDE3.

In addition to infiltration of inflammatory cells we also measured the activation state of the eosinophils at 24h after allergen challenge. Though with all treatment groups, EPO activity in the lavage fluid tended to be diminished, significance was just reached with the lower concentration of Org 20241 only. Furthermore, there is no realtionship between the number of eosinophils of the individual treatment groups and the EPO activity levels in het lavage fluid. In a previous study, using the same animal model, in which the relationship between bronchial hyperreactivity, eosinophil number and EPO activity in the lavage fluid was followed in time, the relationship between eosinophil counts and EPO activity levels found at 6h after allergen challenge was no longer present at 24h either (Santing et al., 1994b).

In conclusion, the results of this study indicate that inhaled PDE inhibitors afford protection against acute histamine- and allergen-induced bronchoconstriction and the development of allergen-induced hyperreactivity (both after the early and late asthmatic
reaction), predominantly through inhibition of PDE4. Remarkably, for inhibition of inflammatory cell infiltration, particularly of eosinophils, both PDE3 and PDE4 inhibition appears to be required.

REFERENCES


