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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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Abstract

In a guinea pig model of allergic asthma, we investigated the effects of the selective phosphodiesterase inhibitors rolipram (phosphodiesterase 4-selective), Org 9935 (phosphodiesterase 3-selective) and Org 20241 (dual phosphodiesterase 4/phosphodiesterase 3-selective), administered by aerosol inhalation in approximately equipotent bronchodilatory doses, on allergen-induced early and late asthmatic reactions, airway hyperreactivity and airway inflammation. Using ovalbumin-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 4,5-dihydro-6-(5,6-dimethoxybenzo[*b*]thien-2-yl-5-methyl-3(2*H*)pyridazinone (Org 9935) and 10 and 100 mM *N*-hydroxy-4-(3,4-dimethoxyphenyl)-thiazole-2-carboximidamide HCl (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 early asthmatic reaction from 0 to 6 h following allergen-provocation. The severity of the late asthmatic reaction was also significantly inhibited by rolipram ($P < 0.05$) and Org 9935 ($P < 0.05$). Allergen-induced airway hyperreactivity to inhaled histamine after the early reaction, at 6 h after ovalbumin challenge, was strongly reduced by rolipram ($P < 0.05$) and completely prevented by the two other phosphodiesterase inhibitors; in addition, airway hyperreactivity after the late asthmatic reaction, at 24 h, was abolished in all treatment groups. Bronchoalveolar lavage performed at 24 h 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 phosphodiesterase 3-, phosphodiesterase 4- and dual phosphodiesterase 3/phosphodiesterase 4-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 phosphodiesterase 4; in contrast, for significant reduction of eosinophil infiltration, both phosphodiesterase 3 and phosphodiesterase 4 inhibition seems to be required. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Phosphodiesterase inhibitor; Allergic asthma; Bronchodilatory property; Anti-inflammatory property; Airway hyperreactivity

1. Introduction

Theophylline has been used in the treatment of asthma for over 50 years, while about 20 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, which currently consists of 11 distinct families (most of them having different isoforms), based on substrate specificity, kinetic properties, sensitivity to selective inhibitors, the effect of allosteric modulators and amino acid sequences (Beavo, 1995; Dousa, 2000; Fawcett et al., 2000). Since the distribution of these subtypes is different for various tissues, subtype-selective

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phosphodiesterase 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 phosphodiesterase 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; Giembycz, 2000).

In airway smooth muscle from animals and man the presence of at least five phosphodiesterase subtypes (phosphodiesterase 1, 2, 3, 4 and 5) has been demonstrated (Torphy and Cieslinsky, 1989; Shahid et al., 1991; De Boer et al., 1992; Torphy et al., 1993). In human airways selective inhibitors of phosphodiesterase 3 and phosphodiesterase 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 phosphodiesterase 3 and phosphodiesterase 4 inhibitors. Furthermore, in other species it has been found, both in vitro and in vivo, that respiratory smooth muscle tone is regulated by both phosphodiesterase 3 and phosphodiesterase 4 (Harris et al., 1989; Heaslip et al., 1991). In addition, phosphodiesterase 4 and to a lesser extent phosphodiesterase 3 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 phosphodiesterase 4, the predominant isoform, attenuates superoxide anion generation, opsonised zymosan-induced respiratory burst, and leukotriene B_4 generation (Dent et al., 1991; Souness et al., 1991; Banner et al., 1996). In addition, rolipram (a phosphodiesterase 4-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 phosphodiesterase inhibitor) abolished allergen-induced eosinophilia and neutrophilia (Elwood et al., 1995). Furthermore, it has been demonstrated that phosphodiesterase 4-selective inhibitors suppress tumor necrosis factor-α (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 phosphodiesterase 4 inhibition (Essayan et al., 1997).

Some reports have indicated that a combined inhibition of phosphodiesterase 3 and phosphodiesterase 4 may be more effective than single inhibition of either isoenzyme. Thus, in T-lymphocytes combined inhibition of phosphodiesterase 3 and phosphodiesterase 4 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 lipopolysaccharide-induced release of TNF-α is only completely inhibited by a combination of rolipram and motapizone (phosphodiesterase 3-selective) (Schudt et al., 1995). Furthermore, in guinea pig tracheal preparations only a combination of phosphodiesterase 3 and phosphodiesterase 4 inhibition causes complete relaxation of carbachol induced contractions (Harris et al., 1989). These findings indicate that mixed phosphodiesterase 3- and phosphodiesterase 4-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 phosphodiesterase inhibitors theophylline (non-selective), rolipram (phosphodiesterase 4-selective) and Org 20241 (dual phosphodiesterase 3- and phosphodiesterase 4-selective) (Nicholson et al., 1995) were observed on the allergen-induced increase in bronchial reactivity both after the early and after the late asthmatic reaction, at 6 h and at 24 h after allergen provocation, respectively, and on airway inflammation at 24 h after allergen challege (Santing et al., 1995); a phosphodiesterase 3-selective inhibitor was not used in that study, however. In the present study we now investigated the effects of rolipram, Org 9935 (phosphodiesterase 3-selective) and Org 20241, administered by aerosol inhalation in bronchodilating doses, on allergen-induced early and late asthmatic reactions, bronchial hyperreactivity after these reactions, and on influx and activation of inflammatory cells.

2. Methods

2.1. 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 immunoglobine E (IgE)-sensitised to ovalbumin at 3 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 (P_{pl}) 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.

2.2. Measurement of airway function

Airway function was assessed by on-line measurement of P_{pl} 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, Bilthoven, The Netherlands), P_{pl} 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).

2.3. 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 2 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.

2.4. Provocation protocols

To determine the effectiveness against histamine-induced bronchoconstriction, 30 min after a control histamine PC_{100}-measurement the lowest concentration of the phosphodiesterase inhibitor (rolipram: 25 μM; Org 9935: 1.0 mM; Org 20241: 1.0 mM) was nebulized for 15 min. After another 15 min a second histamine PC_{100}-determination was performed, starting at 2 concentration steps below the initial PC_{100}. Using 10-fold increased concentrations of the phosphodiesterase inhibitor this procedure was two times repeated after 30 min. PC_{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 min after an initial histamine PC_{100}-measurement, a concentration of histamine was nebulized 10% above the PC_{100}-value. The resulting increase in P_{pl} was used as control value. After another 30 min the selected concentrations of the phosphodiesterase inhibitors were nebulized for 15 min (rolipram: 2.5 mM; Org 9935: 100 mM; Org 20241: 10.0 and 100 mM). Subsequently, at 20, 40, 60, 90, 120, 150, 180, 240, 300, 360, 480, 600, 720 min after treatment, the PC_{110} concentration of histamine was administered again and the resulting increase in P_{pl} was expressed as percent inhibition of the control value.

The effectiveness of the phosphodiesterase inhibitors against allergen-induced early and late phase reactions, bronchial hyperresponsiveness and airway inflammation was determined by administering an aerosol of the phosphodiesterase inhibitors for 15 min, 1 h 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^2, where they could eat and drink ad libitum. To establish the change in airway reactivity at 6 h (between the early and late asthmatic response;

Santing et al., 1992, 1994a) and at 24 h after allergen provocation, the animals were placed in the provocation cage and the PC_{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 early and late asthmatic reaction) were used in this study protocol, since bronchial hyperreactivity is significantly more pronounced in these animals (Santing et al., 1994a).

2.5. Bronchoalveolar lavage procedure

At 24 h after the second allergen provocation animals were anaesthetised with 20 mg ml^{-1} 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 $200 \times g$ for 10 min at 4 °C. The supernatants of the first fractions were rapidly frozen for additional determination of eosinophil peroxidase 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.

2.6. Eosinophil peroxidase assay

The eosinophil peroxidase activity in the supernatant of the first bronchoalveolar lavage fraction, as an indication of eosinophil activation, was analysed according to the kinetic assay described by White et al. (1991), which is based on the oxidation of *o*-phenylenediamine by eosinophil peroxidase in the presence of hydrogen peroxide (H_2O_2). Substrate was made by dissolving 0.018% H_2O_2 and 16 mM *o*-phenylenediamine 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 s 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.

2.7. 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 24 h before and 6 and 24 h after the allergen provocation, respectively (PC_{100} ratio pre/post-allergen challenge). The magnitude of the allergen-induced early and late asthmatic reactions was expressed as the area under the P_{pl}-time curve (AUC) from 0 to 6 h and from 8 to 24 h after allergen provocation, respectively, as calculated by trapezoid integration (Santing et al., 1994a).

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

2.8. Preparation of the drug solutions

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

2.9. Chemicals

Histamine hydrochloride, ovalbumin (grade III), aluminum hydroxide, *o*-phenylenediamine dihydrochloride, and May–Grünwald and Giemsa stain were obtained from Sigma (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-4*H*-1.3-thiazine-hydrochloride, methylparaben) from Bayer (Leverkusen, Germany), and RPMI-1640 medium and Hanks balanced salt solution (HBSS) from Gibco Life Technologies (Praisley, Scotland). 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).

3. Results

3.1. Effectiveness against histamine-induced bronchoconstriction

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

The effectiveness of the phosphodiesterase inhibitors against histamine-induced airway obstruction in sensitized

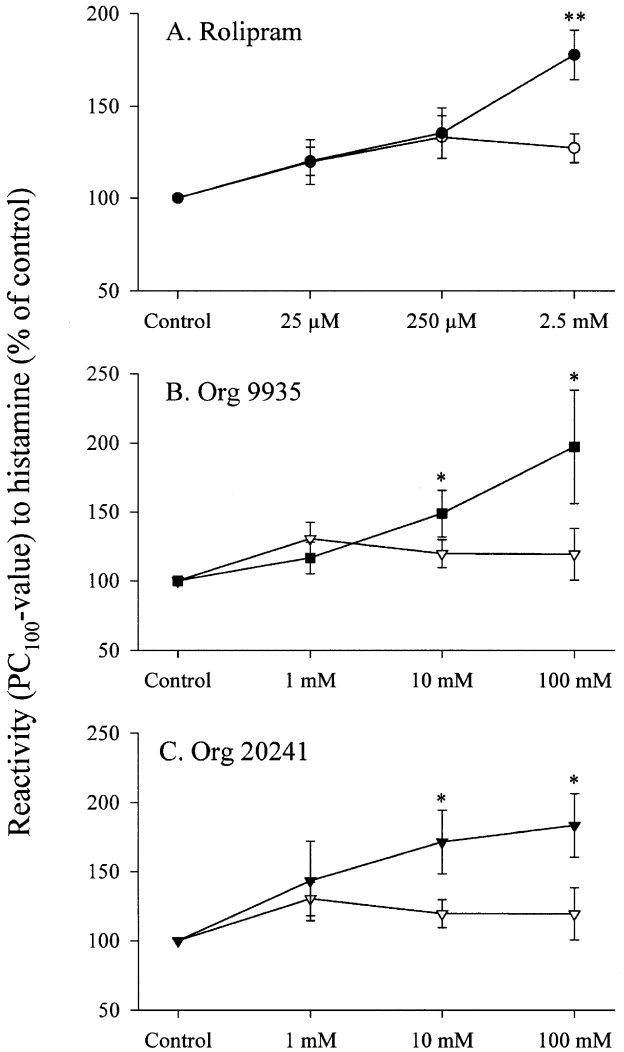

Fig. 1. Protection against histamine-induced bronchoconstriction by inhalation of the phosphodiesterase-inhibitors rolipram, Org 9935, and Org 20241. Nebulizer concentrations used are presented at the abscissa. Open symbols represent data from the corresponding vehicles (saline in case of rolipram; soja–lecithine in saline in case of Org 9935 and Org 20241). Results are presented as percentage of control PC_{100}-values. Data are presented as mean ± S.E.M. from 5–7 experiments. Statistical analysis: paired Student's *t*-test, compared to control; $^{*}P < 0.05$; $^{**}P < 0.01$.

guinea pigs as expressed as a percentage of the PC_{100}-value determined before inhalation of the phosphodiesterase inhibitor is depicted in Fig. 1. For rolipram (phosphodiesterase 4-selective) only the highest concentration (2.5 mM) caused a significant inhibition of histamine-induced bronchoconstriction (1.8-fold increase of the control PC_{100}, $P < 0.01$). Time-matched, vehicle (saline) administrations were without significant effect.

The phosphodiesterase 3-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 phosphodiesterase 3/4-selective) induced a 1.8-fold ($P < 0.05$) and a 1.9-fold ($P < 0.05$) inhibition, respectively. Soja–lecithine 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.

3.2. Duration of inhibition of histamine-induced bronchoconstriction

The duration of the effect of the phosphodiesterase inhibitors against histamine-induced bronchoconstriction was determined by monitoring the increase in P_{pl} induced by a fixed dose of aerosolised histamine (PC_{110}) at several time points after single administration of the phosphodiesterase inhibitor (Fig. 2).

The protective effect of 2.5 mM rolipram against histamine-induced bronchoconstriction remained significant for 90 min; 180 min after rolipram administration the effect had vanished in all 6 animals. The duration of the inhibitory effect of 100 mM Org 9935 was 120 min; 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 min;

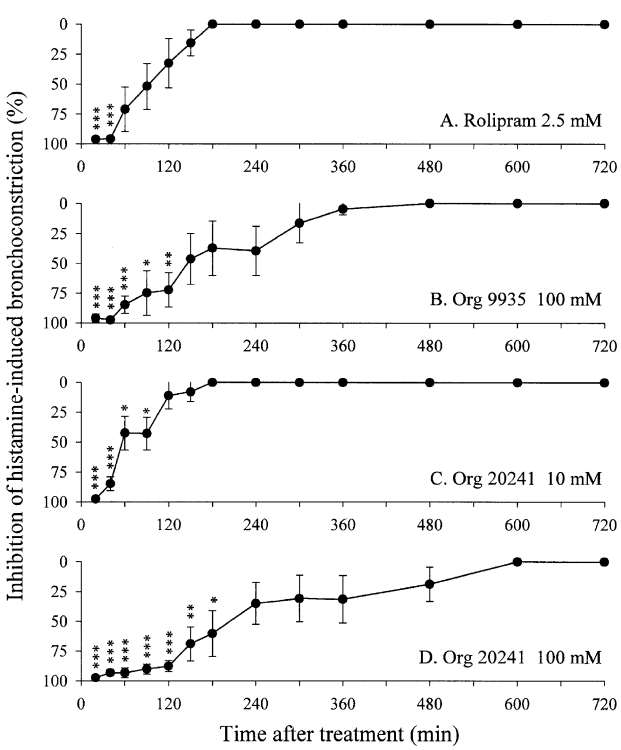

Fig. 2. Time-course of the protection against histamine-induced bronchoconstriction following single inhalation values of selected concentrations of rolipram (2.5 mM), Org 9935 (100 mM) and Org 20241 (10 and 100 mM). Results are presented as percentage inhibition of the individual pre-drug values. Data are expressed as mean ± S.E.M. from 5–6 experiments. Statistical analysis: paired Student's *t*-test, compared to control; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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

Treatment	Early asthmatic reaction (EAR)		Late asthmatic reaction		Maximal height EAR	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Saline	3476 ± 1313	3182 ± 1212	9517 ± 1842	9526 ± 3261	228 ± 76	218 ± 97
Rolipram (2.5 mM)	3460 ± 623	1178 ± 273[a]	5251 ± 1252	1343 ± 654[a]	179 ± 34	92 ± 31[a]
Soja–lecithine	2808 ± 608	1610 ± 546	11,831 ± 1966	6678 ± 1560	164 ± 24	122 ± 33
Org 9935 (100 mM)	4402 ± 728	1271 ± 583[a]	11,036 ± 1218	4992 ± 474[a]	261 ± 44	89 ± 30[a]
Org 20241 (10 mM)	3918 ± 725	2281 ± 891	8206 ± 2634	4952 ± 1654	266 ± 48	172 ± 59[b]
Org 20241 (100 mM)	2656 ± 630	567 ± 491[a]	11,367 ± 3133	6750 ± 1149	244 ± 47	68 ± 16[b]

Inhalation of vehicle (saline or soja–lecithine), rolipram, Org 9935 and Org 20241 1 h before the second allergen provocation. Data are presented as area under P_{pl} time–response curve between 0 and 6 h after allergen provocation for the early asthmatic reaction and between 8 and 24 h after allergen provocation for the late asthmatic reaction. Maximal height of the early asthmatic reaction is expressed as percent change of P_{pl}. Data represent mean values ± S.E.M. for 4–7 animals.

[a] Statistical analysis: Student's *t*-test for paired observations (before/after treatment), $P < 0.05$.

[b] Statistical analysis: Student's *t*-test for paired observations (before/after treatment), $P < 0.01$.

histamine-induced bronchoconstrictions returned to control levels in all seven animals at 180 min 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 min. Inhibition was still present in some animals at 480 min after inhalation of this concentration of Org 20241.

3.3. Effectiveness against allergen-induced early and late asthmatic reactions and bronchial hyperreactivity

The data of the allergen-induced early and late reactions (presented as AUC) before and after treatment with vehicle and the phosphodiesterase 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.

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

The effect of the phosphodiesterase inhibitors on the bronchial hyperreactivity measured at 6 h (after the early and late asthmatic reactions) and 24 h (after the late asthmatic reaction) after allergen provocation is presented in Fig. 3. All phosphodiesterase inhibitors significantly suppressed the allergen-induced increase in bronchial reactivity at 6 h after allergen provocation; remarkably, in the animal groups treated with Org 9935 (100 mM) and Org

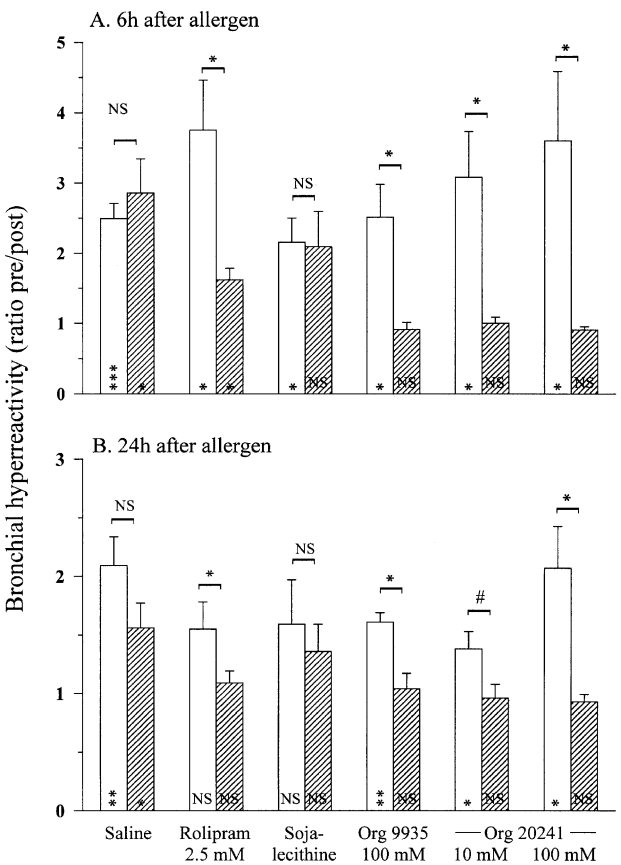

Fig. 3. Effect of inhalation of saline, rolipram 2.5 mM, soja–lecithine in saline, Org 9935 100 mM, Org 20241 10 and 100 mM on allergen-induced bronchial hyperreactivity to histamine at 6 h (upper panel) and 24 h (lower panel) after allergen provocation. Open bars: first (control) provocation, hatched bars: second (treated) provocation. Data (PC_{100} ratio's pre/post-allergen provocation) are presented as means ± S.E.M. from 5–6 experiments. Statistical analysis: paired Student's *t*-test, compared to PC_{100} before allergen-provocation (presented in the bars) and compared to PC_{100} at 6 and 24 h after the first allergen provocation (presented on top of the bars); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P = 0.06$; N.S.: not significant.

20241 (both concentrations), the development of the early bronchial hyperreactivity was completely prevented.

The development of bronchial hyperreactivity observed after the late asthmatic reaction 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 ($P = 0.06$).

3.4. Effects on allergen-induced inflammation

The analysis of the cellular contents of the bronchoalveolar lavage obtained 24 h after the second allergen provocation is depicted in Fig. 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 phosphodiesterase inhibitors (except the lower concentration of Org 20241) but did not reach significance. Lymphocyte and macrophage numbers were only decreased significantly by Org 9935.

The effect on the eosinophil peroxidase activity measured in the bronchoalveolar lavage-fluid is shown in Fig. 5. After inhalation of all phosphodiesterase inhibitors, a tendency towards decreased eosinophil peroxidase activity was observed; however, only with the lower Org 20241 concentration did this reduction reach statistical significance.

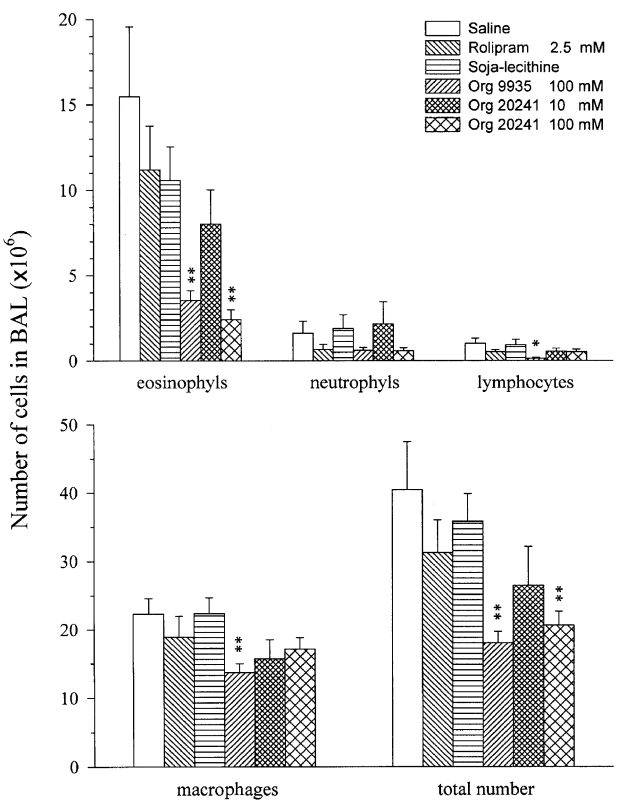

Fig. 4. Total and differential inflammatory cell numbers in bronchoalveolar lavage performed at 24 h after the second allergen provocation. Results are presented as millions of cells retrieved. Data presented as mean ± S.E.M. from 6–8 animals are given. Statistical analysis: unpaired Student's *t*-test, compared to appropriate control; $^{*}P < 0.05$; $^{**}P < 0.01$.

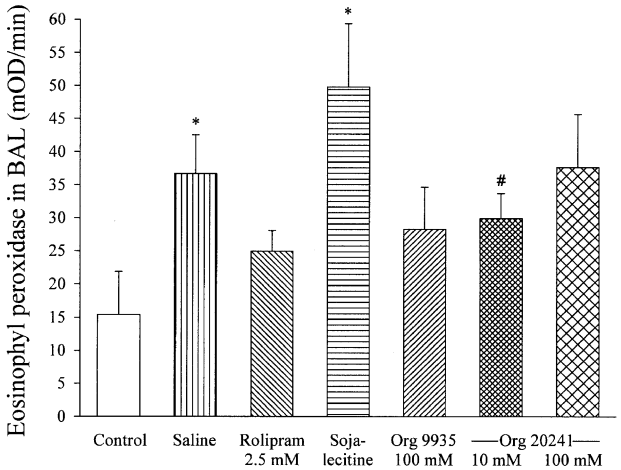

Fig. 5. Eosinophil peroxidase activity in bronchoalveolar lavage at 24 h after the second allergen provocation. Control data are from sensitised, non-challenged animals. Results are presented as mOD/min from the first bronchoalveolar lavage supernatant. Data are presented as mean ± S.E.M. from 4–7 animals. Statistical analysis: unpaired Student's *t*-test. $^{*}P < 0.05$, compared to control; $^{\#}P < 0.05$ compared to soja–lecithine.

4. 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 early and late asthmatic reactions, bronchial hyperreactivity, and airway inflammation were studied (Santing et al., 1995). Marked inhibition of the hyperreactivity to histamine, both after the early and late asthmatic reaction, were observed with all drugs, whereas the severity of the early and late asthmatic reactions 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 phosphodiesterase 3-, a phosphodiesterase 4- and a dual phosphodiesterase 3/phosphodiesterase 4-inhibitor, when given in bronchodilationg doses through the inhalational route. To select approximately equipotent nebulizer concentrations of the drugs, different concentrations of each phosphodiesterase 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 per-

formed before and after inhalation of the drugs. The results showed that rolipram (phosphodiesterase 4-selective) at 2.5 mM, Org 9935 (phosphodiesterase 3-selective) at 100 mM and Org 20241 (dual phosphodiesterase 3/4-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 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 phosphodiesterase inhibitor is nebulized into a 9-l 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 phosphodiesterase 4' (Harris et al., 1989; Souness and Rao, 1997). Remarkably, Org 20241, having a more shallow dose–effect relationship than Org 9935, was essentially equipotent to the phosphodiesterase 3 inhibitor, despite the fact that Org 9935 is at least 100-fold selective for inhibiting phosphodiesterase 3 compared to phosphodiesterase 4, whereas Org 20241 is a dual inhibitor with some selectivity for phosphodiesterase 4 (Nicholson et al., 1995). Indeed, the high potency and selectivity of Org 9935 for inhibiting the phosphodiesterase 3 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 phosphodiesterase inhibitors is mainly due to inhibition of phosphodiesterase 4.

The duration of the protection by the selected concentrations of the phosphodiesterase 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 phosphodiesterase 3 inhibition, recent data in guinea pig airways by Spina et al. (1998) have shown that the duration of action of phosphodiesterase inhibitors is independent of isoenzyme-selectivity.

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

The late asthmatic reaction was also significantly inhibited by rolipram and Org 9935, whereas Org 20241 did not provide a significant protection.

Although the inhibition of the early asthmatic reaction may partially be attributed to the direct bronchodilating effects of the phosphodiesterase inhibitors, the inhibition of the late asthmatic reaction 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 bronchial hyperreactivity, both after the early asthmatic reaction (at 6 h) and after the late asthmatic reaction (at 24 h after allergen provocation) by all phosphodiesterase inhibitors. It should be mentioned that at 6 h, 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 late asthmatic reaction 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 that inhibition of both phosphodiesterase 3 and phosphodiesterase 4 activity (by Org 9935 and the higher concentration of Org 20241), rather than of phosphodiesterase 4 alone, is involved in the reduction of eosinophil migration would emerge.

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 phosphodiesterase 3/4 inhibitor compared to selective phosphodiesterase 3 or phosphodiesterase 4 inhibitors. However, other studies have clearly demonstrated antigen-induced infiltration of eosinophils to be decreased by selective phosphodiesterase 4 inhibitors, including rolipram, RO 20-1724 and RP 73401 (Underwood et al., 1993, 1997; Raeburn et al., 1994; Ortiz et al., 1996; Danahay and Broadley, 1997). In these studies, the phosphodiesterase 4 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 phosphodiesterase inhibitors would reduce the generation of chemotactic mediators involved in eosinophil recruitment. Since both phosphodiesterase 3 and phosphodiesterase 4 are present in lung mast cells (Weston et al.,

1997), dual inhibition of both isoenzymes could be more effective than phosphodiesterase 4 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 phosphodiesterase 3.

In addition to infiltration of inflammatory cells we also measured the activation state of the eosinophils at 24 h after allergen challenge. Though with all treatment groups, eosinophil peroxidase 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 relationship between the number of eosinophils of the individual treatment groups and the eosinophil peroxidase activity levels in the lavage fluid. In a previous study, using the same animal model, in which the relationship between bronchial hyperreactivity, eosinophil number and eosinophil peroxidase activity in the lavage fluid was followed in time, the relationship between eosinophil counts and eosinophil peroxidase activity levels found at 6 h after allergen challenge was no longer present at 24 h either (Santing et al., 1994b).

In conclusion, the results of this study indicate that inhaled phosphodiesterase 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 phosphodiesterase 4. Remarkably, for inhibition of inflammatory cell infiltration, particularly of eosinophils, both phosphodiesterase 3 and phosphodiesterase 4 inhibition appears to be required.

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