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Modulation of eotaxin formation and eosinophil migration by selective inhibitors of phosphodiesterase type 4 isoenzyme

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1 This study was undertaken to investigate the possible contribution of the blockade of eotaxin generation to the anti-eosinophilotactic effect of phosphodiesterase (PDE) type 4 inhibitors. In some experiments, the putative synergistic interaction between PDE type 4 inhibitors and the β2-agonist salbutamol was also assessed.

2 Sensitized guinea-pigs aerosolized with antigen (5% ovalbumin, OVA) responded with a significant increase in eotaxin and eosinophil levels in the bronchoalveolar lavage fluid (BALF) at 6 h. Eosinophil recruitment was inhibited by both PDE type 4 inhibitors rolipram (5 mg kg^{-1}, i.p.) and RP 73401 (5 mg kg^{-1}, i.p.) treatments. In contrast, only rolipram inhibited eotaxin production.

3 Sensitized rats intrapleurally challenged (i.pl.) with antigen (OVA, 12 μg $cavity^{-1}$) showed a marked eosinophil infiltration at 24 h, preceded by eotaxin generation at 6 h. Intravenous administration of a rabbit anti-mouse eotaxin antibody (0.5 mg kg^{-1}) significantly reduced allergen-evoked eosinophilia in this model.

4 Local pretreatment with rolipram (40 μg $cavity^{-1}$) or RP 73401 (40 μg $cavity^{-1}$) 1 h before challenge reduced eosinophil accumulation evaluated in the rat pleural effluent, but only the former was active against eotaxin generation. The inhibitors of PDE type 3 (SK&F 94836) and type 5 (zaprinast) failed to alter allergen-evoked eosinophil recruitment in rats.

5 Local injection of β2-agonist salbutamol (20 μg $cavity^{-1}$) inhibited both eosinophil accumulation and eotaxin production following pleurisy. The former was better inhibited when salbutamol and rolipram were administered in combination.

6 Treatment with rolipram and RP 73401 dose-dependently inhibited eosinophil adhesion and migration *in vitro*. These effects were clearly potentiated by salbutamol at concentrations that had no effect alone.

7 Our findings indicate that although rolipram and RP 73401 are equally effective in inhibiting allergen-induced eosinophil infiltration only the former prevents eotaxin formation, indicating that PDE 4 inhibitors impair eosinophil accumulation by mechanisms independent of eotaxin production blockade.

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Abbreviations: OVA, 5% ovalbumin; PDE, phosphodiesterase

Introduction

Eosinophils are considered as effector cells in some pathologies associated with eosinophilia, including immediate hypersensitivity reaction and other allergic diseases (Busse *et al*., 1996). They can release a wide range of products such as intragranular toxic proteins and eicosanoid derivatives which may cause dysfunction and tissue damage (for review see Rothenberg, 1998). Evidence shows that increased levels of cationic proteins were detected in the bronchoalveolar lavage or sputum of patients with asthma (Gleich, 1990). In allergic inflammatory reactions, eosinophils appear in large numbers infiltrated into the affected tissues, although the mechanism responsible for their attraction is poorly understood. Several factors including lipid mediators and cytokines have been implicated in tissue eosinophil recruitment (for review see Giembycz & Lindsay, 1999). Moreover, in the past few years, a large family of small chemotactic cytokines with four conserved cysteines linked by disulfide bonds has been discovered and termed chemokines (for review see Luster, 1998). Among them, a more selective recruitment of eosinophils is likely to occur in response to the members of the CC chemokine subfamily of which eotaxin is the most likely candidate mediator (Griffiths-Johnson *et al*., 1993; Jose *et al*., 1994).

Intracellular increase of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) has been shown to mediate a variety of

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anti-inflammatory effects (Bourne *et al*., 1974). Rises in intracellular cyclic AMP are usually transient, since it is rapidly hydrolysed to adenosine 5′-monophosphate (AMP) by phosphodiesterase (PDE) enzymes. PDEs are currently identified as an eleven member family of isoenzymes (Francis *et al*., 2000) and PDE type 4 has been shown to be the most prominent in immune and inflammatory cells including eosinophils (Hall, 1993; Teixeira *et al*., 1997). Selective inhibitors of PDE type 4 were reported as effective compounds in suppressing several eosinophil functions including superoxide generation (Barnette *et al*., 1995), adhesion (Santamaria *et al*., 1997) and migration evaluated in Boyden chamber (Alves *et al*., 1996; Santamaria *et al*., 1997). In addition, systemic treatment with PDE type 4 inhibitors was shown to suppress effectively tissue eosinophil accumulation caused by several stimuli (Lagente *et al*., 1994; Underwood *et al*., 1993; Teixeira *et al*., 1994). Furthermore, β2-agonists, besides their extremely potent bronchodilator activity (Cullum *et al*., 1969), were shown to inhibit infiltration and activation of eosinophils associated with allergen stimulation (Diaz *et al*., 1996) by a mechanism dependent on the increase of cyclic AMP levels, as a consequence of receptor-coupled adenylate cyclase activation (Giembycz & Dent, 1992). The combination of β2-agonists with selective PDE type 4 inhibitors was reported to exert synergistic bronchodilator and anti-inflammatory effects as attested by inhibition of antigen-induced contraction (Planquois *et al*., 1996) and vascular permeability increase (Planquois *et al*., 1998) in guinea-pig trachea, phenomena which contribute to the pathophysiology of allergic diseases such as asthma. As eosinophil accumulation in tissues is a prominent feature of allergic disorders, in the present study we investigated the effect of the administration of the selective PDE type 4 inhibitors rolipram and RP 73401 on two models of allergen-induced eosinophil recruitment and eotaxin generation. In some experiments, the potential synergism between rolipram and the β2-agonist salbutamol was also analysed.

Methods

Animals, sensitization procedure and antigen challenge

Wistar rats (180–250 g) (Oswaldo Cruz Foundation, Rio de Janeiro, Brazil) and Dunkin Hartley guinea-pigs (300–400 g) (Charles River Laboratories, Kent, U.K.), of both sexes, were used in accordance with the guidelines of the Committee on Use of Laboratory Animals of the Home Office (U.K.) and the Oswaldo Cruz Foundation (Brazil). Rats were subcutaneously sensitized with a mixture of ovalbumin (OVA, 50 μg) and $Al(OH)_3$ (5 mg). On day 14, OVA (12 μg cavity^{-1}) was intrapleurally (i.pl.) injected into hypnorm-anaesthetized animals (0.7 mg kg^{-1} fentanyl citrate and 20 mg kg^{-1} fluanisone). Six and 24 h later, the animals were killed with CO_2 followed by cervical dislocation, the pleural cavity was opened and rinsed with 3 ml of PBS containing EDTA (10 mM) as described (Silva *et al*., 1992). For guinea-pigs, the sensitization consisted of an intraperitoneal (i.p.) injection of OVA (10 μg) mixed with $Al(OH)_3$ (5 mg) on days 1 and 15. On day 28, the animals were pretreated with the histamine (H_1) receptor antagonist pyrilamine (10 mg kg^{-1}, i.p.), to avoid fatality, and exposed to aerosolized OVA (1%) for 20 min (Humbles *et al*., 1997). Six hours later, the guinea-pigs were killed with barbiturate overdose and the BAL was performed with 15 ml of PBS + EDTA (10 mM). In both rat and guinea-pig models, the inflammatory effluents were centrifuged at $1000 \times g$ for 10 min and the supernatant was stored at -20°C until assayed for eotaxin. Non-sensitized animals injected with OVA or sensitized animals injected with saline were used as negative controls.

Leucocyte counts

Total leucocytes were counted in a Coulter counter ZM after dilution of the inflammatory fluids (40 μl) with 20 ml of lsoton II plus Zap-oglobin II. The differential analysis was performed under oil immersion objective in cytocentrifuged smears coloured with May–Grunwald–Giemsa stain. Mast cells were counted in Neubauer chamber after dilution of the pleural fluid samples (90 μl) with Toluidine blue dye solution (10 μl) and evaluated as total number including intact and degranulated cells.

Protein quantification

The fluid recovered from the pleural cavity was centrifuged at $1000 \times g$ for 10 min and the total protein content spectrophotometrically quantified (540 nm) in the supernatant, by means of the Biuret technique.

Quantification of eotaxin by ELISA

Eotaxin in rat pleural lavage fluid was measured by ELISA as reported briefly by Bandeira-Melo *et al*. (2000). Unless stated otherwise, all incubations in microtiter plates (Costar, Cambridge, MA, U.S.A.) were for 1 h with 100 μl reagent well^{-1}. Plates were coated overnight with a polyclonal anti-murine eotaxin antibody (R&D Systems, Minneapolis, MN, U.S.A.) and washed (four cycles) with 10 mM potassium phosphate buffer pH 7.5/0.02% thimerosal/0.05% Tween 20. Nonspecific binding sites were blocked with 250 μl 1% bovine serum albumin in PBS and the plates again washed. Recombinant murine eotaxin (Peprotech, 5-640 pM in PBS/ 0.05% thimerosal/ 0.1% BSA) and cell free lavage fluid was added for 1 h before washing with PBS/0.02% thimerosal/ 0.2% Tween 20. Plates were then incubated with a biotinylated goat anti-murine eotaxin (R&D Systems), washed and incubated with a neutravidin-horseradish peroxidase conjugate (Pierce, Chester, U.K.). After the final washing, plates were developed with K-blue substrate (Neogen, Lexington, KY, U.S.A.) for 30 min before stopping the reaction with 0.19 M H_2SO_4 for absorbance reading at 450 nm. Rat eotaxin (97.3% sequence identity with murine eotaxin) (Williams *et al*., 1998; Ishi *et al*., 1998) cross reacts in this assay but there were no detectable cross-reaction with any other chemokine tested including human and guinea-pig eotaxin.

A similar protocol was used to measure guinea-pig eotaxin except that the coating antibody was a murine monoclonal anti-guinea-pig eotaxin, plates were blocked with 1% skimmed milk in PBS and the detector antibody was a rabbit polyclonal anti-guinea-pig eotaxin (Humbles *et al*., 1997) which was then reacted with a horseradish peroxidase-conjugated donkey anti-

rabbit IgG antibody (Amersham Pharmacia, U.K.). The standard guinea-pig eotaxin (5-160 pM) was synthesized by Dr Glen Andrews, Pfizer Central Research, (Groton, CT, U.S.A.). No cross-reaction was detected with any other chemokine tested including human, mouse and rat eotaxin.

Purification of rat eosinophils

Eosinophil purification was performed using Percoll density gradient according to technique described by Martins *et al.* (1989). Briefly, eosinophils were obtained from the peritoneal cavity of anaesthetized normal rats and lavage cells were centrifuged at $1500 \times g$ at 20°C for 18 min. The supernatant was discarded and the pellet was resuspended in RPMI-1640 medium (pH 7.2) containing 30 mM HEPES and 2 mg ml^{-1} sodium bicarbonate. The cells were pooled, the suspension was layered onto discontinuous Percoll gradient and the tubes centrifuged at $2440 \times g$ at 20°C for 30 min. Eosinophils were collected and washed twice with RPMI-1640 medium. Cell viability was evaluated by Trypan blue exclusion. Eosinophils of 85–95% purity and 96% viability were used in the following experiments.

Chemotaxis assay

Migration experiments were performed using 48-well microchemotaxis chamber (Neuro Probe, Inc. U.S.A.) and a Toyo cellulose nitrate filters (3 μm pore) (Richards & McCullough, 1984). Eotaxin (0.1 μM), platelet-activating factor (PAF) (1 μM) and RPMI-1640 medium containing bovine serum albumin were placed in the lower compartment and the eosinophil suspension (2×10^5 cells) placed in the upper compartment of the chamber. To test the interference of PDE 4 inhibitors rolipam and RP 73401 as well as the β2-agonist salbutamol, purified eosinophils were pre-incubated with the drugs or with respective vehicles at 37°C in a 5% CO_2:95% O_2 atmosphere for 30 min. Rolipram and RP 73401 were dissolved at 20% Tween 80 and diluted to the desired concentration with saline, whereas salbutamol was dissolved in saline solution (NaCl, 0.9%). The chamber was incubated at 37°C in a 5% CO_2:95% O_2 atmosphere for 2 h and the filter fixed and stained as described (Richards & McCullough, 1984). Eosinophils migrated at 40 μm from the upper surface of the filter were counted in 15 consecutive high-power fields (HPF) under an immersion objective. All experiments were done in duplicates.

Eosinophil adherence assay

Eosinophil adhesion was analysed as residual eosinophil peroxidase (EPO) activity of adhered cells (Sedgwick *et al.*, 1988). Briefly, flat bottom 96-well plates were pretreated with BSA (0.5%) for 2 h at room temperature and then rinsed with HBSS prior to use. Following the addition of 50 μl of control medium (HBSS Ca^{2+} Mg^{2+}) or PAF (1 μM), purified eosinophils (50 μl of 1×10^6 cells ml^{-1}) were placed in the wells and left incubated for 1 h in a humidified 5% CO_2 atmosphere. One hundred μl of original eosinophil suspension was placed in empty wells as measure of total EPO activity. After three cycles of gently washings with HBSS containing FCS (1%), 50 μl of HBSS containing 0.5% hexadecyltrimethylamonium bromide was added and the suspension freeze/thawed three times. Fifty μl of the samples were placed in empty wells. Then, 100 μl of the substrate (2.2 mM O-phenylenediamine and 1 mM hydrogen peroxide in 0.1 M TRIS-HCl buffer, pH 8.0) was added and, after a 30 min-incubation at room temperature, 50 μl of 4M H_2SO_4 was added to stop the reaction and the absorbance read at 492 nm. Percentage of adhesion was calculated as the ratio between the O.D. of residual cells and O.D. of total cells $\times$ 100.

Treatment

Guinea-pigs were intraperitoneally treated with the selective PDE type 4 inhibitors rolipram (5 mg kg^{-1}) and RP 73401 (5 mg kg^{-1}), and the PDE type 3 inhibitor trequinsin (1.25 mg kg^{-1}) administered 1 h before antigen challenge. In the case of rats, rolipram (10–40 μg $cavity^{-1}$) and RP 73401 (10–40 μg $cavity^{-1}$) as well as the inhibitors SK&F 94836 (PDE type 3) and zaprinast (PDE type 5) (40 μg $cavity^{-1}$) were injected i.pl., 1 h before the allergen. In another set of experiments, rats were treated with the β2-agonist salbutamol (20 μg $cavity^{-1}$) either alone or in combination with rolipram, 1 h before pleural stimulation. Control animals received the same volume of the vehicle.

Rat eotaxin protein was shown to have 97.3% identity with mouse eotaxin (Ishi *et al.*, 1998). Thus, to analyse the role of eotaxin in the pleural eosinophil recruitment in sensitized rats, animals were treated with rabbit polyclonal antibody anti-murine eotaxin (0.5 mg kg^{-1}) or the control rabbit IgG (0.5 mg kg^{-1}) intravenously 30 min before the antigen challenge.

Materials

Ovalbumin (grade V) was purchased from Biochemika Fluka (Switzerland). Rolipram was a generous gift from the Institut de Recherche Jouveinal (France), SK&F 94836 was from Smith-Kline Beecham (U.K.) and RP 73401 from Rhône-Poulenc Rorer Ltd (U.K.). Bovine serum albumin, pirilamine, zaprinast, salbutamol and trequinsin were purchased from Sigma Chemical Co (St. Louis, MO, U.K.). The rabbit polyclonal antibody anti-murine eotaxin was kindly given by Dr Nick Luckacs (University of Michigan, U.S.A.). All solutions were freshly prepared immediately before use.

Statistical analysis

The data are reported as means $\pm$ s.e.m. and were statistically analysed by ANOVA followed by Newman–Keuls–Student's *t*-test. Probability values (P) of 0.05 or less were considered significant.

Results

Effect of systemic treatment with rolipram and RP 73401 on antigen-evoked eosinophil recruitment and eotaxin generation in the guinea-pig lung

Sensitized guinea-pigs were challenged with aerosolized ovalbumin and BAL samples collected 6 h post-challenge. In line with previous reports (Jose *et al.*, 1994; Humbles *et al.*, 1997), we confirmed here that allergen challenge is associated with eotaxin production and a marked eosinophil

infiltration into the airway lumen (Figure 1). The treatment with either rolipram (5 mg kg^{-1}) or RP 73401 (5 mg kg^{-1}), administered 1 h before challenge, clearly abolished the eosinophil recruitment as shown in Figure 1A and C respectively. In contrast, only rolipram was active against eotaxin generation (Figure 1B and D). In addition, the PDE type 3 inhibitor trequinsin (1.25 mg kg^{-1}, i.p.) failed to alter both eosinophil influx into the airways or eotaxin production (Figure 1E and F).

Eosinophil influx and eotaxin production following allergen-mediated pleurisy

Confirming previous reports (Lima *et al*., 1990; Silva *et al*., 1992), we observed that sensitized rats challenged with an i.pl. injection of specific antigen (ovalbumin, 12 μg $cavity^{-1}$) responded with marked eosinophilia, which was first noted within 12 h and peaked 24 h post-challenge (Figure 2A). The antigen-induced eosinophilia was preceded by an increase in the eotaxin levels in the pleural effluent, noted 3 and 6 h post-challenge and reducing thereafter (Figure 2B). Furthermore, as shown in Table 1, antigen-induced eosinophilia was sensitive to pretreatment with a neutralizing polyclonal antibody raised against murine eotaxin, administered by intravenous route 30 min before challenge.

Effect of local PDE type 4, 3 and 5 inhibitors on antigen-induced pleural eosinophil recruitment and eotaxin generation

Local pretreatment with the PDE 4 inhibitor rolipram (10 and 40 μg $cavity^{-1}$), 1 h before antigen stimulation, significantly reduced the pleural eosinophil accumulation noted at 24 h (Figure 3A) as well as eotaxin generation noted at 6 h post-challenge (Figure 3B). It is noteworthy that rolipram (40 μg $cavity^{-1}$) did not modify the basal pleural eosinophil population which was $1.40 \pm 0.09 \times 10^6$ cells $cavity^{-1}$ ($n=7$) in vehicle-treated animals and $1.33 \pm 0.22 \times 10^6$ cells $cavity^{-1}$ ($n=8$) (mean $\pm$ s.e.m.) in rolipram-treated animals. Differently from what was observed with rolipram, treatment of

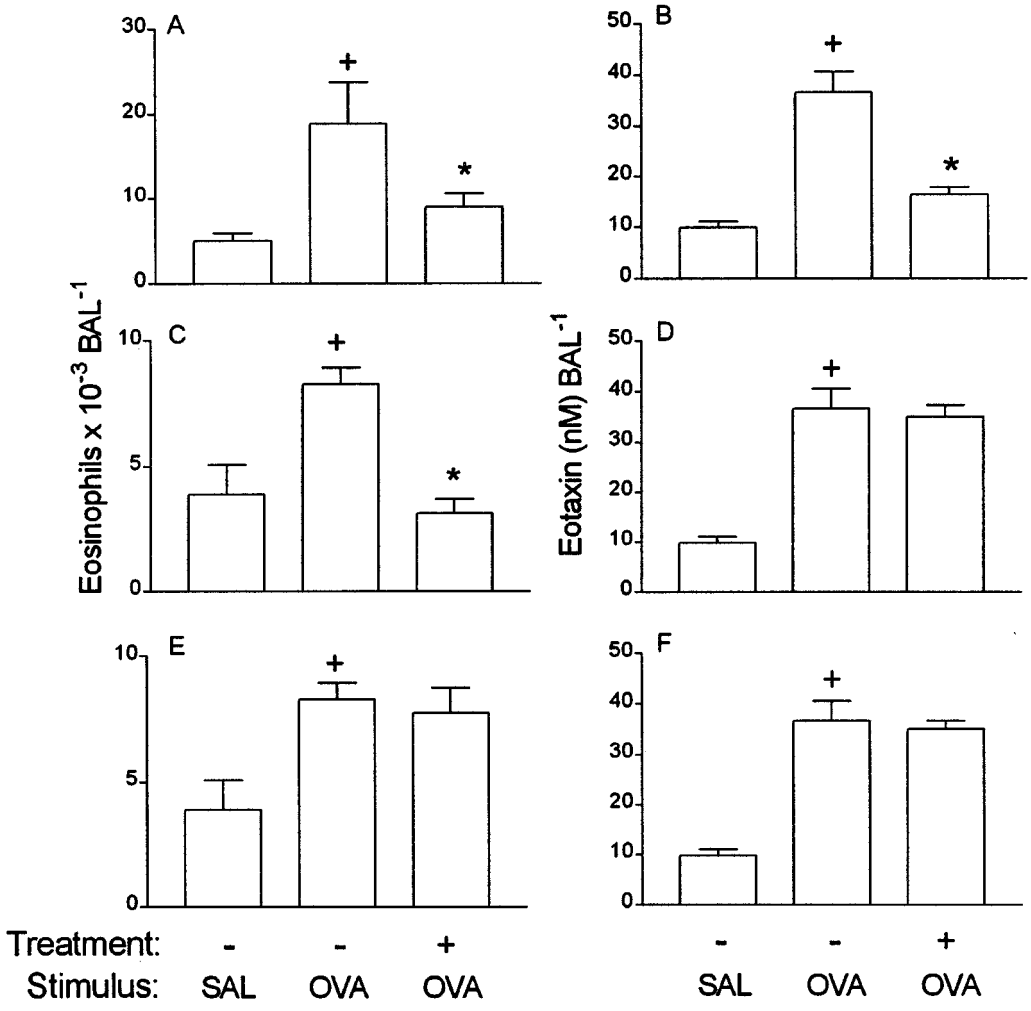

Figure 1 Effect of inhibitors of PDE 4 rolipram (5 mg kg^{-1}) and RP 73401 (5 mg kg^{-1}) and PDE 3 trequinsin (1.25 mg kg^{-1}) on eosinophil accumulation (A, C, E) and eotaxin generation (B, D, F) in the bronchoalveolar lavage (BAL) of sensitized guinea-pigs 6 h after aerosolized ovalbumin 1% (OVA). Sensitized animals challenged with saline (SAL) were used as controls. Each point represents the mean $\pm$ s.e.mean of at least six animals. $^{+}P<0.05$ as compared to sensitized non-challenged animals; $^{*}P<0.05$ as compared to the sensitized challenged animals.

sensitized rats with either inhibitors of PDE 3 SK&F 94836 or PDE 5 zaprinast had no effect on the antigen-induced pleural eosinophil infiltration (Table 2).

The compound RP 73401 has been shown to be a more selective and less toxic PDE 4 inhibitor in several systems, as compared to rolipram (Souness *et al*., 1995; Teixeira *et al*., 1997). In our conditions RP 73401 locally administered was

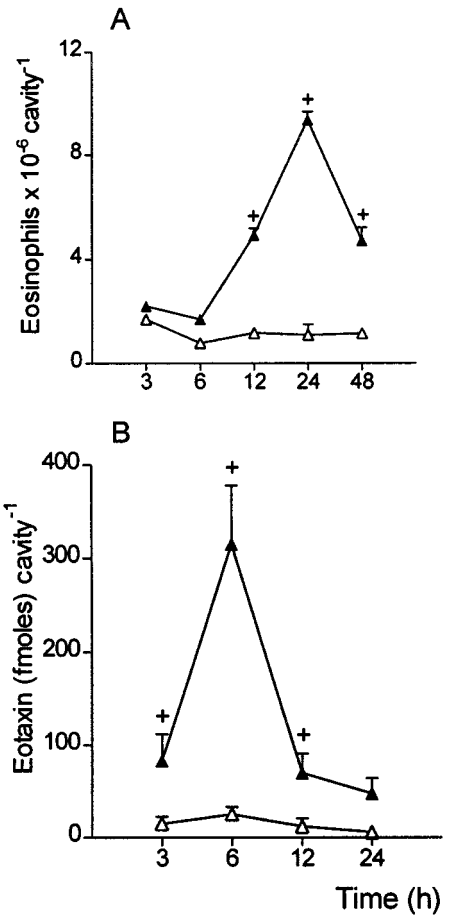

Figure 2 Time course of (A) eosinophil accumulation and (B) eotaxin generation in the pleural cavity of sensitized rats challenged with antigen (OVA, 12 μg cavity^{-1}) (closed symbols). Non-sensitized rats (open symbols) injected with OVA were used as controls. Each point represents the mean$\pm$s.e.mean of at least seven animals. $^{+}P<0.05$ as compared to the non-sensitized animals.

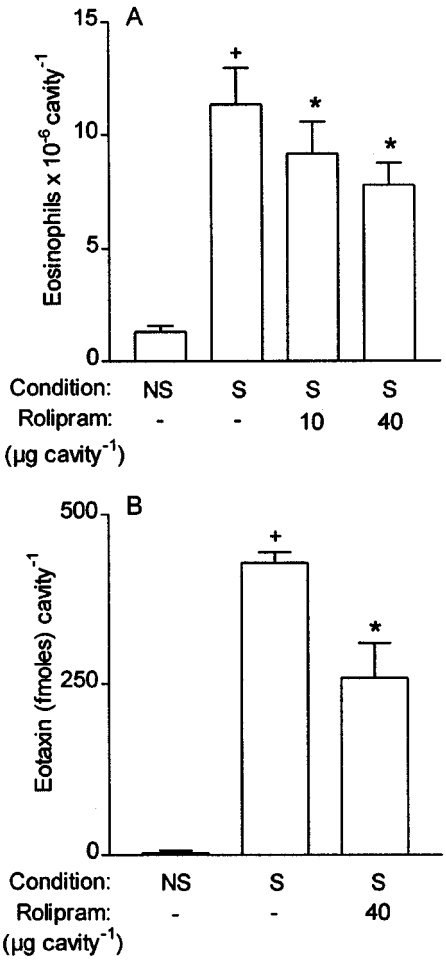

Figure 3 Effect of the local treatment with rolipram (10 and 40 μg cavity^{-1}) on the (A) eosinophil accumulation and (B) eotaxin generation in the pleural cavity of sensitized rats (S) challenged with antigen (OVA, 12 μg cavity^{-1}). Non-sensitized rats (NS) injected with OVA were used as controls. The analyses of eotaxin formation and eosinophil infiltration were performed at 6 and 24 h post-challenge respectively. Each point represents the mean$\pm$s.e.mean of at least seven animals. $^{+}P<0.05$ as compared to the non-sensitized animals; $^{*}P<0.05$ as compared to the sensitized challenged animals.

Table 1 Inhibitory effect of the anti-eotaxin antiserum on the eosinophil infiltration induced by antigen in the pleural cavity of sensitized rats.

Condition	*Eosinophils* (i.v.)	*Animals* ($\times 10^{6\ \text{cavity}-1}$)	(n)
Non-Sensitized	Saline	1.27$\pm$0.12	6
Sensitized	Saline	5.18$\pm$0.73^{+}	5
Sensitized	Normal serum	4.71$\pm$0.73	4
Sensitized	α-Eotaxin serum	3.49$\pm$0.20*	6

The anti-eotaxin polyclonal antibody (0.5 mg kg^{-1}) or the control rabbit IgG (0.5 mg kg^{-1}) were intravenously injected 30 min before the challenge (ovalbumin, 12 μg cavity^{-1}), and the pleural fluid analysis was performed 24 h later. Animals injected with saline were used as negative control. Values represent the mean$\pm$s.e.mean. $^{+}P<0.05$ as compared to non-sensitized challenge rats; $^{*}P<0.05$ as compared to sensitized challenge rats.

Table 2 Lack of effect of selective PDE inhibitors SK&F 94836 (type 3) and zaprinast (type 5) on the antigen-induced pleural eosinophil recruitment in sensitized rats.

Drug	*Dose* (μg/cavity)	*Eosinophils* ($\times 10^{6\ \text{cavity}-1}$)	*Animals* (n)
SK&F 94836	0	7.52$\pm$0.65^{+}	6
	40	8.67$\pm$1.09	7
Zaprinast	0	7.32$\pm$0.61^{+}	6
	40	7.58$\pm$0.62	8

The compounds were locally administered 1 h before ovalbumin (12 μg cavity^{-1}) and the analysis was performed 24 h later. The number of eosinophils of non-sensitized rats injected with antigen was 1.31$\pm$0.17$\times 10^{6}$ cavity^{-1} ($n=8$). Values represent the mean$\pm$s.e.mean of at least seven animals. $^{+}P<0.05$ as compared to non-sensitized challenge rats.

able to inhibit antigen-mediated pleural eosinophilia (Figure 4A) but did not modify the concomitant elevation in the eotaxin production (Figure 4B).

Effect of combination of rolipram with salbutamol on antigen-induced eosinophil migration in the pleurisy model

Previous studies of our group have demonstrated that the local administration of the β2-agonist salbutamol prevented, in a dose-dependent manner, plasma leakage, neutrophil and eosinophil accumulation after allergen challenge in rats (Diaz *et al*., 1996). Here we found that the inhibition of allergen-induced eosinophil accumulation in the pleural cavity by salbutamol (20 μg cavity^{-1}) occurred in parallel with the blockade of eotaxin generation, as shown in Figure 5A. In addition, we noted that there is a clear synergism between salbutamol and rolipram concerning the blockade of eosinophil infiltration, which could not be reproduced in respect to eotaxin production (Figure 5B).

Lack of effect of rolipram on protein extravasation and mast cell degranulation caused by allergen in the pleurisy system

The local pretreatment with the PDE 4 inhibitor rolipram (40 μg cavity^{-1}), 1 h before stimulation, failed to modify the allergen-evoked plasma leakage and mast cell degranulation noted 30 min after challenge (Table 3).

Effect of the combination of salbutamol with either rolipram or RP 73401 on eosinophil adhesion and chemotaxis in vitro

Experiments conducted in the BSA-coated plate system using purified eosinophils from the peritoneal cavity of naive rats showed that 1 h incubation with PAF (1 μM) led to an enhancement of cell adhesion from 28.3$\pm$2.7% (n=3) to

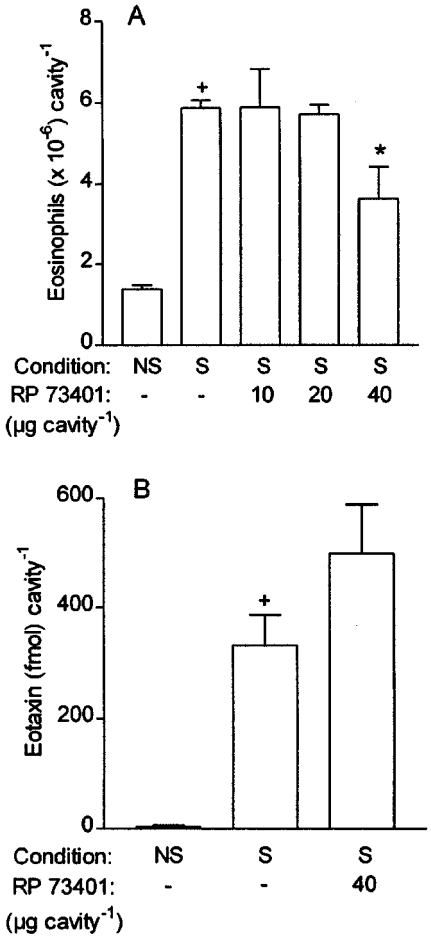

Figure 4 Effect of the local treatment with RP 73401 (10 and 40 μg cavity^{-1}) on the (A) eosinophil accumulation and (B) eotaxin generation in the pleural cavity of sensitized rats (S) challenged with antigen (OVA, 12 μg cavity^{-1}). Non-sensitized rats (NS) injected with OVA were used as controls. The analyses of eotaxin formation and eosinophil infiltration were made at 6 and 24 h post-challenge, respectively. Each point represents the mean$\pm$ s.e.mean of at least seven animals. $^{+}P<0.05$ as compared to the non-sensitized animals; $^{*}P<0.05$ as compared to the sensitized challenged animals.

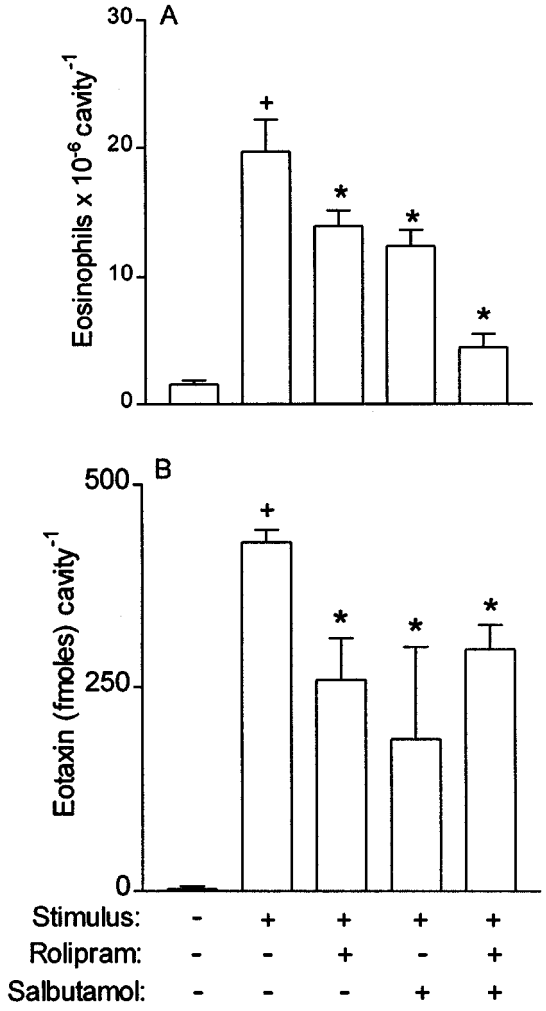

Figure 5 Effect of the local treatment with the combination of rolipram (40 μg cavity^{-1}) and the β2-agonist salbutamol (20 μg cavity^{-1}) on the (A) eosinophil accumulation and (B) eotaxin generation, in the pleural cavity of sensitized rats (S) challenged with antigen (OVA, 12 μg cavity^{-1}). Non-sensitized rats (NS) injected with OVA were used as controls. The analyses of eotaxin formation and eosinophil infiltration were performed at 6 and 24 h post-challenge respectively. Each point represents the mean$\pm$s.e.mean of at least seven animals. $^{+}P<0.05$ as compared to the non-sensitized animals; $^{*}P<0.05$ as compared to the sensitized challenged animals.

Table 3 Lack of effect of rolipram on the antigen-induced mast cell degranulation and protein leakage in pleural cavity of sensitized rats

Condition	*Treatment*	*Total protein* (mg)	*Intact mast cells* (10^3 cells activity^{-1})	*Animals* (n)
Non-sensitized	Saline	3.89 ± 0.30	517.28 ± 39.93	7
Sensitized	Saline	$27.85 \pm 4.23^{+}$	$52.52 \pm 21.32^{+}$	8
Sensitized	Rolipram	21.30 ± 3.09	88.70 ± 14.93	6

Rolipram was locally administered 1 h before ovalbumin (12 μg cavity^{-1}) and the analysis performed 30 min later. Values represent the mean $\pm$ s.e.mean. $^{+}P<0.05$ as compared to non-sensitized challenge rats.

$52.5 \pm 6.6\%$ ($n=4$), in the absence and presence of PAF, respectively. As illustrated in Table 4, pretreatment with either rolipram (0.01 – 10 μM) or RP 73401 (1 nM – 0.1 μM) dose-dependently inhibited eosinophil adhesion. The combination of 80 μM salbutamol with either rolipram (0.1 μM) or RP 73401 (1 nM) potentiated the inhibitory effect of these drugs, under conditions where salbutamol was ineffective (Figure 6). In another set of experiments using the 48-well Boyden chamber system (Richards & McCullough, 1984), we noted that the pretreatment of eosinophils with either rolipram (0.1 – 10 μM) or RP 73401 (0.1 and 1 nM) inhibited 1 μM PAF-induced eosinophil chemotaxis in a dose-dependent manner, whereas salbutamol (100 μM) was also inactive (Figure 7). The combination of 80 μM salbutamol with 0.1 μM rolipram led to synergistic blockade of eosinophil migration induced by either 1 μM PAF or 0.1 μM eotaxin *in vitro*, as shown in Figure 8.

Table 4 Effct of the selective PDE 4 inhibitors rolipram and RP 73401 on eosinophil adhesion *in vitro*

Treatment	*Dose* (μM)	*Inhibition* (%)	(n)
Rolipram	0.01	25.74 ± 1.89*	3
	0.1	43.14 ± 8.18**	3
	10	43.43 ± 2.17**	3
RP 73401	0.001	18.65 ± 4.26	3
	0.01	24.15 ± 2.77*	4
	0.1	45.51 ± 4.90**	4

Eosinphils were incubated for 30 min with the compounds and the cell adhesion was evaluated 1 h after PAF stimulation (1 μM). Values represent the mean $\pm$ s.e.mean of experiment done in triplicate. $*P<0.05$ as compared to PAF-stimulated eosinophils.

Discussion

In this study, the effects of inhibitors of cyclic nucleotide PDE on allergen-evoked eotaxin upregulation and subsequent eosinophil recruitment were investigated. Using two distinct models of allergic inflammation, we demonstrated that the selective PDE type 4 inhibitors rolipram and RP 73401 prevented eosinophil infiltration into the guinea-pig airways and rat pleural space. In contrast, only rolipram was active against allergen-evoked eotaxin production, as attested by ELISA quantification. Experiments presented here also demonstrated that both eotaxin formation and eosinophil recruitment were equally sensitive to the β2-agonist salbutamol. The combination of rolipram and salbutamol synergistically inhibited allergen-induced eosinophil accumulation *in vivo*, without improving the blockade of eotaxin generation presented by each treatment alone. In addition, salbutamol clearly potentiated the inhibitory effect of rolipram *in vitro*. Altogether these data indicate that the impairment of eotaxin production is not a central property in the anti-eosinophilotactic activity of PDE type 4 inhibitors.

PDE enzymes provide the only known pathway for the enzymatic inactivation of the cyclic-nucleotide second messengers, making them pivotal regulators of signal transduction mechanisms (Beavo & Reifsnyder, 1990; Giembycz & Dent, 1992). The anti-inflammatory properties of PDE type 4 inhibitors have been emphasized in several cell systems such as blockade of chemotaxis, adhesion, and degranulation in neutrophils and eosinophils and the blockade of production of a range of inflammatory mediators (Nicholson & Shahid, 1994). These properties render PDE type 4 inhibition an obvious candidate therapy for asthma and other allergic disorders (Spina *et al*., 1998; Higgs, 1998; Barnette & Underwood, 2000).

It has already been established that PDE type 4 inhibitors, including rolipram, attenuate skin (Cooper *et al*., 1999) and lung eosinophilia as well as airway hyperresponsiveness triggered by virus and allergen challenge (Lagente *et al*., 1994, Ikemura *et al*., 2000; Kung *et al*., 2000). The CC chemokine eotaxin is a powerful chemoattractant which has been shown to drive eosinophil recruitment in numerous studies involving human and animal systems (e.g. Humbles *et al*., 1997; Sanz *et al*., 1998). Therefore, the chance does exist that PDE type 4 inhibitors are impairing eosinophil recruitment and airway hyperresponsiveness by preventing eotaxin production. In fact, in a system of TNF-α-induced eotaxin release from human airways smooth muscle cells rolipram was shown to inhibit eotaxin release (Pang & Knox, 2001). Previous studies revealed that there is a causative relationship between increased levels of immunoreactive eotaxin and eosinophil influx into the lumen of sensitized guinea-pigs after challenge with aerosolized ovalbumin (Jose *et al*., 1994; Humbles *et al*., 1997). Using the same system, we found here that the intraperitoneal treatment with PDE type 4 inhibitor rolipram, but not type 3 inhibitor trequinsin, abolished both eosinophil accumulation and eotaxin protein upregulation noted in the BAL fluid 6 h post-allergen challenge. In contrast, allergen-evoked eotaxin production was clearly resistant to the selective PDE type 4 inhibitor RP 73401, under conditions where the eosinophil accumulation was entirely suppressed. These findings suggest that the blockade of eosinophil accumulation by PDE type 4 inhibitors is not necessarily accounted for by blockade of eotaxin production.

Similar data were obtained using the rat allergic pleurisy system. In line with our previous study (Bandeira-Melo *et al*.,

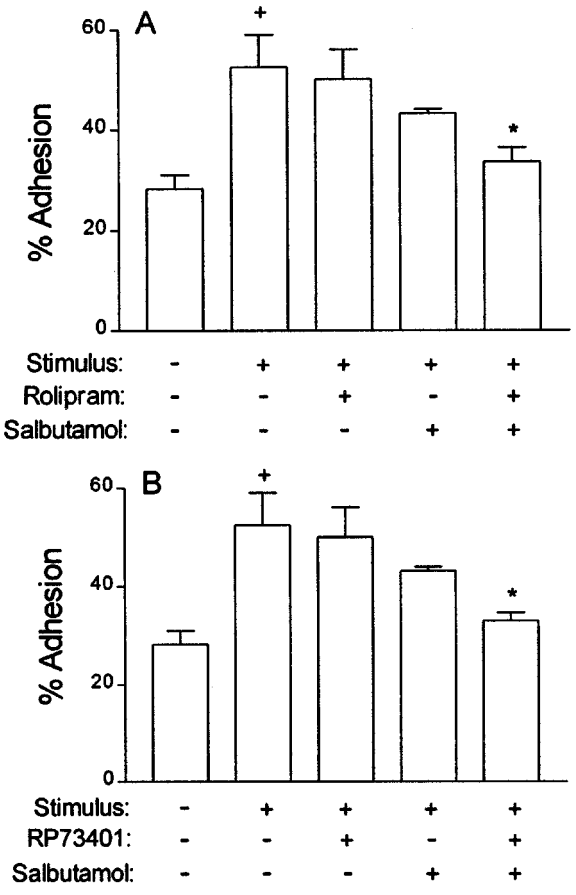

Figure 6 Effect of the PDE 4 inhibitor rolipram (0.1 μM) (A) and RP 73401 (1 nM) (B) in combination with the β2-agonist salbutamol (80 μM) on eosinophil adhesion to BSA coated plate. Cells were pre-incubated with vehicle or the drugs for 30 min prior to PAF stimulation (1 μM). Each point represents the mean$\pm$s.e.mean of two separate experiments done in quadruplicate. $^{+}P<0.05$ as compared to non-stimulated eosinophils; $^{*}P<0.05$ as compared to salbutamol- or rolipram-treated PAF-stimulated eosinophils.

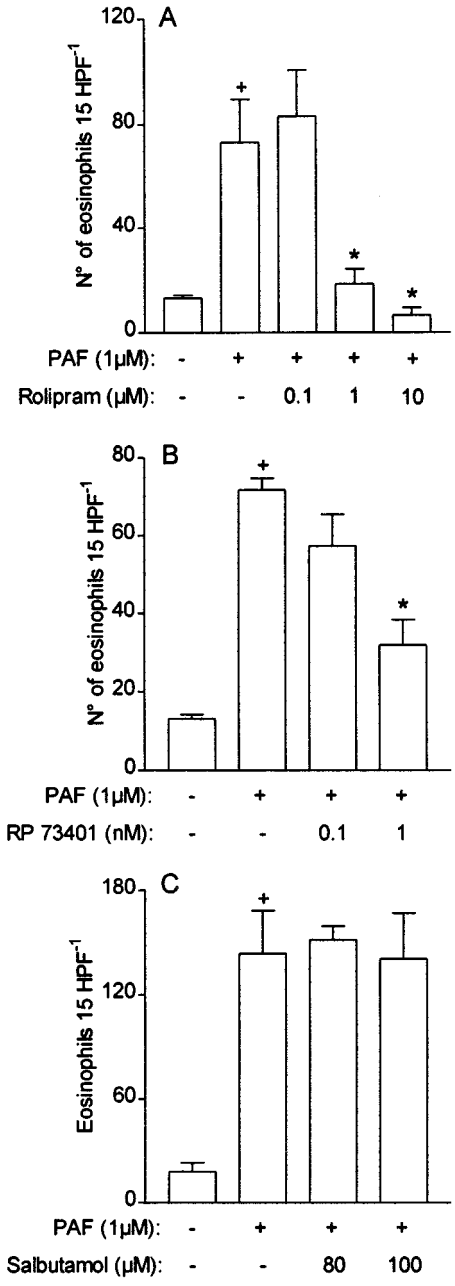

Figure 7 Effect of the PDE 4 inhibitors rolipram (0.1 – 10 μM) (A), RP 73401 (0.1 and 1 μM) (B) and of the β2-agonist salbutamol (80 and 100 μM) (C) on eosinophil chemotaxis induced by PAF (1 μM). Cells were pre-incubated with vehicle or drugs for 30 min prior to stimulation. Each point represents the mean$\pm$s.e.mean of seven separate experiments done in duplicate. $^{+}P<0.05$ as compared to non-stimulated eosinophils; $^{*}P<0.05$ as compared to vehicle-treated PAF-stimulated eosinophils.

2000), we demonstrated here that allergen-induced late eosinophil influx into the pleural space of sensitized rats was preceded by a marked increase in eotaxin production. As reported for the guinea-pig airway inflammation system (Humbles *et al*., 1997), eotaxin concentration in the pleural effluent peaked within 6 h reducing thereafter. In addition, we showed that allergen evoked pleural eosinophilia was sensitive to the treatment with a neutralizing antibody to eotaxin, reinforcing the causative relationship between both phenomena. Using this model, we further reassessed whether a family of selective inhibitors of PDE isoenzymes protect eotaxin and eosinophilia following allergen challenge. Consistently with what was shown in guinea-pigs, rolipram and RP 73401, now locally administered, significantly inhibited eosinophil accumulation, but only the former prevented eotaxin generation. Differently from our data, Teixeira *et al*. (1994) previously reported that co-injection of rolipram with antigen had little effect on the infiltration of ^{111}In-labelled eosinophils in the guinea-pig skin, although PAF-induced eosinophilia was partially inhibited by the local administration of rolipram. The better efficacy of local rolipram noted in our conditions may involve differences in the doses, treatment scheme and experimental models used. Overall, our findings indicate that either systemic or local PDE type 4 inhibition is associated with a marked inhibition of antigen-induced eosinophil accumulation in the tissues. The efficacy of both ways of administering the drug also suggest that the cellular site of action that could account for the their suppressive effect on eosinophil recruitment may be the same. It seems reasonable to speculate that systemic treatment with PDE type 4 inhibitors may lead to an increase

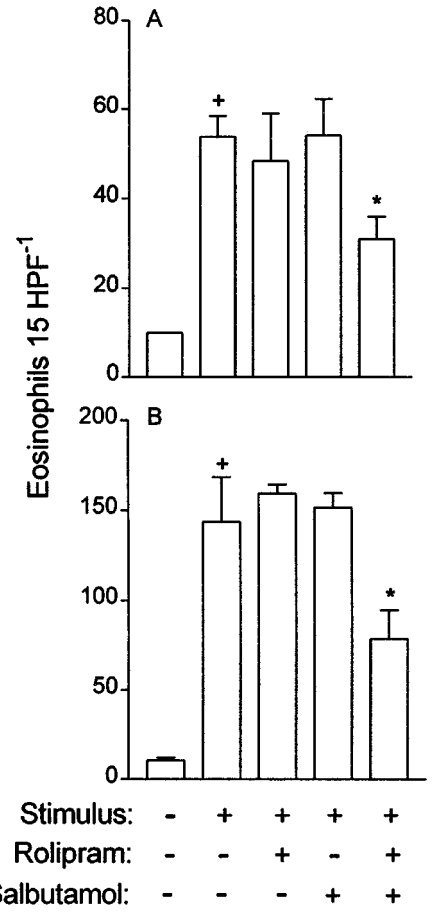

Figure 8 Effect of the PDE 4 inhibitor rolipram (0.1 μM) in combination with the β2-agonist salbutamol (80 μM) on eosinophil chemotaxis induced by PAF (1 μM) (A) or eotaxin (0.1 μM) (B). Cells were pre-incubated with vehicle or the drugs for 30 min prior to stimulation. Each point represents the mean$\pm$s.e.mean of four separate experiments done in duplicate. $^{+}P<0.05$ as compared to non-stimulated eosinophils; $^{*}P<0.05$ as compared to salbutamol- or rolipram-treated PAF-stimulated eosinophils.

in the levels of cyclic AMP in circulating eosinophils. However, we cannot rule out the possibility that some of the locally injected PDE 4 type inhibitors may also be reaching migrating eosinophils. In this line of thinking, we have previously demonstrated that eosinophils are themselves important sites of action for the inhibitory effects of systemically and locally administered cyclic AMP-elevating agents (Teixeira & Hellewell, 1997). Pre-treatment with the inhibitors of PDE type 3 (SK&F 94836) and PDE type 5 (zaprinast) failed to alter allergen-evoked eosinophil recruitment in sensitized rats. These observations suggest that eosinophil accumulation is indeed sensitive to PDE type 4 inhibitors rather than to PDE types 3 and 5, which is in line with previous reports (Lagente *et al*., 1994; Teixeira *et al*., 1994). One possible explanation for these findings is that rolipram does not prevent eotaxin formation *via* blockade of the PDE type 4 isoenzyme (Kambayashi *et al*., 1995). Furthermore, whatever the mechanism underlying the inhibitory effects observed, it is clear, from our study, that rolipram is capable of inhibiting allergen-evoked eotaxin generation under conditions where RP 73401 is not. A possibility exists that PDE type 4 isoforms may exhibit distinct sensitivity to pharmacological inhibition and/or different isoforms may be present on the cell target for eotaxin generation as compared to those present on eosinophils. However, there is no published demonstration that rolipram or RP 73401 can distinguish the multiple PDE type 4 isoforms (Manning *et al*., 1999). Alternatively, the failure of RP 73401 to inhibit eotaxin generation may be either due to a barrier, preventing the access and interaction of the inhibitor with the enzyme (Souness *et al*., 1991) or to a distinct interaction with PDE type 4 as compared to rolipram. Additionally, a comparative study performed by Souness *et al*. (1995), using pig aortic smooth muscle cells and guinea-pig eosinophils, clearly showed that rolipram and RP 73401 can exert their effects on cyclic AMP hydrolysis through distinct interactions with PDE type 4.

Next, we aimed to assess the influence on eotaxin production of the β2-agonist salbutamol, which is known to increase intracellular levels of cyclic AMP in several systems *via* adenilate cyclase activation (Cullum *et al*., 1969). Using the allergic pleurisy model, we have previously reported that the local administration of salbutamol, at doses varying from 10 to 40 μg cavity^{-1}, significantly inhibited allergen-induced eosinophil accumulation into the pleural space (Diaz *et al*., 1996). The phenomenon was prevented by the β-adrenoceptor antagonist propranolol, indicating that the protective activity of salbutamol was entirely mediated by β2-adrenoceptors (Diaz *et al*., 1996). It is polemic in the literature whether or not β2-agonists could potentially exert an antiinflammatory action in addition to their recognized ability to induce bronchodilatation (Howarth *et al*., 1985). However, Li *et al*. (1999) found a significant reduction in airway eosinophilic infiltrate in human biopsies, as assessed by immunostaining. In our conditions, it was demonstrated that the salbutamol-dependent blockade of eosinophil accumulation was accompanied by a significant inhibition of eotaxin generation. Our data are in line with those from Pang & Knox (2001), who evidenced the ability of salbutamol to inhibit TNF-α-induced eotaxin release from smooth muscle cells. This implies that like rolipram, salbutamol is also able to prevent eotaxin production *in vivo*, which prompted us to investigate a potential synergism between rolipram and salbutamol as regards eosinophil influx blockade. It is noteworthy that the β2-agonist isoprenaline worked in a synergistic manner with rolipram in guinea-pig macrophages to elevate the cyclic AMP content (Kelly *et al*., 1998). In another study, low concentrations of β2-agonists, including salbutamol, increased the relaxant effect of PDE type 4 inhibitors on antigen-induced contraction of guinea-pig isolated trachea (Planquois *et al*., 1996). In the current conditions, we did find an additive reduction in eosinophilic infiltrate in pleural space by locally combining rolipram and salbutamol. However, the extent to which eotaxin was inhibited by the combined treatment was not significantly different from those noted after their administration alone. The lack of synergism between rolipram and salbutamol concerning the blockade of eotaxin formation is in line with the interpretation that these drugs may be acting in distinct compartments to inhibit the elevation of eotaxin and the influx of eosinophils in order to elevate intracellular levels of cyclic AMP in the cell source of eotaxin. Furthermore, this result suggests a dissociation between the synergistic blockade of the eosinophil accumulation and the inhibition of eotaxin formation.

It is noteworthy that rolipram failed to alter either plasma leakage or mast cell degranulation stimulated by the intrapleural allergen challenge in sensitized rats, indicating that, at least in this model, the effect of rolipram was largely restricted to the decrease of eosinophil infiltration and not related to an effect on the mast cells. On the other hand, it should be borne in mind that the *in vitro* exposure of rat and human eosinophils to rolipram decreases eosinophil migration stimulated by PAF, LTB_4 or eotaxin (Alves *et al*., 1996; Santamaria *et al*., 1997). In this study, we demonstrated that salbutamol itself did not modify eosinophil migration evoked by either PAF or eotaxin *in vitro* but clearly improved the inhibitory effect of rolipram, in line with what was observed in the *in vivo* assay. It is noteworthy that RP 73401 also dose-dependently inhibited *in vitro* eosinophil chemotaxis, clearly showing to be more potent than rolipram as previously reported (Souness *et al*., 1995; Cooper *et al*., 1999). Previous studies reported PDE 4 inhibitors have the ability to block leukocyte adhesion as well as expression of cell adhesion molecules (Berends *et al*., 1997). Thus, inhibition of eosinophil adhesion could also explain the ability of PDE 4 inhibitors to suppress eosinophil recruitment after allergen stimulation. In order to test this hypothesis, we used an *in vitro* BSA-covered plate system, a model shown to be associated with the expression of CD18 (Walsh *et al*., 1990). We found that incubation with either rolipram or RP 73401 was able to block dose-dependently PAF-induced eosinophil adhesion. Here, RP 73401 was again more potent than rolipram. In addition to CD18-mediated interactions, VLA-4 is a member of the $\beta 1$ integrin family that also has been shown to contribute importantly to the eosinophil trafficking (Giembycz & Lindsay, 1999, Larbi *et al*., 2000). Thus, we cannot exclude the possibility that PDE type 4 inhibitors could be acting on the expression of VLA-4 to inhibit tissue eosinophil mobilization. Experiments are now underway in order to clarify better this latter possibility. Alternatively, inhibition of endothelial cell adhesion molecule expression could be a putative site for the PDE type 4 inhibitors. In fact, Blease *et al*. (1998) demonstrated the ability of PDE type 4 inhibitors to suppress the expression of VLA-4 ligand VCAM-1 on microvascular endothelial cells *in vitro*. Similarly to what was noted for chemotaxis, the combination of salbutamol with rolipram or RP 73401 greater inhibited PAF-induced eosinophil adhesion than each inhibitor alone. Our data are in accordance with others which showed a synergistic effect between cyclic AMP-elevating agonists and rolipram in inhibiting adhesion molecule expression (Berends *et al*., 1997; Blease *et al*., 1998). Taken overall, our results are consistent with the interpretation that the direct effect of PDE type 4 inhibitors on the eosinophil adhesion and locomotory functions greatly contributes to their ability to impair tissue eosinophil accumulation.

In conclusion, our findings indicate that allergen-evoked eotaxin generation is sensitive to treatment with either rolipram or salbutamol, a mechanism that may contribute to the blockade of tissue eosinophil accumulation exerted by these drugs. However, this mechanism does not underlie the inhibition of the eosinophil infiltration produced by RP 73401, indicating that the suppression of eotaxin generation is not equally important to the anti-eosinophilotactic action of PDE type 4 inhibitors in general. In addition, these data provide evidence that the suppression of allergic eosinophilia by both rolipram and RP 73401 may be dependent, at least partially, on a direct blockade of adhesive and migratory properties of eosinophils.

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