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Selective inhibition of phosphodiesterase type IV suppresses the chemotactic responsiveness of rat eosinophils in vitro

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Abstract

Previous studies demonstrated that the selective inhibition of phosphodiesterase type IV suppresses antigen-induced eosinophil infiltration and also downregulates certain eosinophil functions assessed in vitro. In the current study, we compared the effect of selective inhibitors of phosphodiesterase IV with the effect of phosphodiesterase III and V inhibitors, focusing on eosinophil chemotaxis stimulated by platelet-activating factor (PAF) and leukotriene B_4 in a modified Boyden chamber. The effect of β_2-adrenoceptor agonists and forskolin as well as the analogue *N*6-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (Bt_2 cyclic AMP) was also determined. For this purpose eosinophils were obtained by lavage of the peritoneal cavity of normal Wistar rats and purified on Percoll gradients to 85–95% purity. Our results showed that PAF and leukotriene B_4 (0.001–10 μM) elicited a concentration-dependent increase in eosinophil migration with maximal responses observed at 1 μM and 0.1 μM respectively. Pre-incubation with the type IV phosphodiesterase inhibitor, rolipram (1–100 μM), suppressed the chemotactic response triggered by PAF and leukotriene B_4, in association with elevation of eosinophil cyclic AMP, whereas the compounds milrinone and SK&F 94836 (type III selective) as well as zaprinast (type V selective) were ineffective. The β_2-adrenoceptor agonists salbutamol and salmeterol (1–100 μM) did not alter the intracellular levels of cyclic AMP and also failed to inhibit the eosinophil response. Moreover, incubation of eosinophils with the adenylate cyclase activator forskolin (1–100 μM), while inducing a discrete increase in cyclic AMP, markedly inhibited PAF- and leukotriene B_4-induced eosinophil chemotaxis. Eosinophils treated with a combination of individually inactive amounts of forskolin plus rolipram significantly inhibited the eosinophil migration elicited by PAF and leukotriene B_4, but did not change cyclic AMP baseline levels. Though only at the highest concentration tested (100 μM), the analogue Bt_2 cyclic AMP abolished the eosinophil chemotaxis. Thus we conclude that the direct inhibitory effect of phosphodiesterase IV inhibitors on eosinophil chemotaxis may account for their suppressive activity on tissue eosinophil accumulation following antigen challenge.

Keywords: Peritoneal eosinophil; Migration, in vitro; Phosphodiesterase inhibition; β_2-Adrenoceptor agonist

1. Introduction

Cyclic nucleotides are thought to act as second messengers in the regulation of the functional activity of different cell types (Kaliner and Austen, 1974). In the majority of inflammatory cells elevation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) concentrations is associated with suppression of some cellular responses including cytotoxicity, degranulation, mediator release and chemotaxis (Undem et al., 1990; Derian et al., 1994). One way to increase cyclic AMP levels is to inhibit its degradation, which has been shown to depend on a range of structurally related enzymes (phosphodiesterases), biochemically characterized by their distinct substrate specificities and regulatory characteristics (Beavo et al., 1994). Five distinct isoenzyme families have now been distinguished and in the majority of inflammatory cells, the predominant phosphodiesterase isoenzyme is type IV. Selective inhibitors of the various phosphodiesterases are now available, which permits the pharmacological manipulation of several phys-

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iopathological processes including allergic inflammatory diseases (Nicholson and Shahid, 1994).

Eosinophils are considered important contributors to the triggering and/or perpetuation of allergic inflammation (Frigas and Gleich, 1986; Kroegel et al., 1994). They are commonly recruited into sites undergoing hypersensitivity reactions or immunologically mediated diseases (Gleich et al., 1987; Silva et al., 1992) by a mechanism partially dependent on cell adhesion and chemotaxis (Walsh et al., 1993). Pretreatment with the non-selective phosphodiesterase inhibitor theophylline is shown to be effective against eosinophil infiltration after antigen challenge (Sanjar et al., 1990; Sullivan et al., 1994), an effect also shared by type IV selective phosphodiesterase inhibitors (Dent et al., 1991). Indeed, phosphodiesterase IV inhibition by compounds such as rolipram and Ro 20-1724 was shown to suppress eosinophil infiltration in the guinea-pig lung induced by antigen challenge, PAF exposure or IL-5 injection (Lagente et al., 1994, 1995). Thus, in this study we compared the effect of selective phosphodiesterase IV inhibitors with the effects of phosphodiesterase III and V inhibitors on the eosinophil chemotaxis using the Boyden chamber system. The efficacy of agents capable of increasing intracellular levels of cyclic AMP, including adenylate cyclase activators such as salbutamol, salmeterol and forskolin, as well as the analogue Bt_2 cyclic AMP, was also considered.

2. Materials and methods

2.1. Purification of rat eosinophils

Eosinophil purification was performed using Percoll density gradients (Gartner, 1980) with some modifications. Briefly, eosinophils were obtained from the peritoneal cavity of ether-anaesthetised normal Wistar 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 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) buffer and 2 mg/ml sodium bicarbonate. The cells were pooled and the suspension was layered onto a discontinuous Percoll gradient, which consisted of 2 ml of 72% and 3 ml of 56% solutions of Percoll carefully overlayered. Tubes were centrifuged at $2400 \times g$ at 20°C for 30 min and eosinophils were collected from the interface between the Percoll bands and washed twice with RPMI-1640 medium. The final cell suspension (4 million eosinophils/ml) was kept in 1 ml of RPMI-1640 medium containing ovalbumin (4 mg/ml). Cells were counted with a Neubauer chamber and differential analyses were performed with cytospin preparations stained with May-Grunwald-Giemsa dye. Cell viability was evaluated by trypan dye exclusion. Eosinophils of 85–95% purity and 96% viability were used in the following experiments.

2.2. Chemotaxis assay

Migration experiments were performed using a 48-well microchemotaxis chamber (Neuro Probe, USA) and Toyo cellulose nitrate filters (3 μm pore) according to the technique described by Richards and McCullough (1984). Leukotriene B_4 (0.001–0.1 μM), platelet-activating factor (PAF) (0.001–0.1 μM) and RPMI-1640 medium containing bovine serum albumin (29 μl) were placed in the lower compartment and 50 μl of the eosinophil suspension (200 000 cells) was placed in the upper compartment of the chamber. To test the interference of the phosphodiesterase inhibitors and cyclic AMP-active agents, purified eosinophils were pre-incubated with either drug or with the respective vehicle at 37°C for 30 min, in a 5% CO_2 : 95% O_2 atmosphere. Selective inhibitors of phosphodiesterase type III (milrinone and SK&F 94836), type IV (rolipram and Ro 20-1724) and type V (zaprinast) were used at the concentration range 0.1–100 μM. Bt_2 cyclic AMP and forskolin were tested at similar concentrations and the β_2-adrenoceptor agonists salbutamol and salmeterol at 1–100 μM. All the phosphodiesterase inhibitors and forskolin were dissolved in 20% Tween-80 and diluted to the desired concentration with saline, whereas salbutamol, salmeterol and Bt_2 cyclic AMP were dissolved in saline solution (NaCl, 0.9%). The chamber was incubated for 2 h at 37°C in a 5% CO_2 : 95% O_2 atmosphere and the filter was fixed and stained as described (Richards and McCullough, 1984). Eosinophils that had migrated at 40 μm from the upper surface of the filter were counted in 15 consecutive high-power fields under an immersion objective. All experiments were done in duplicate. Results were expressed as a coefficient migration (cf), which was calculated according to the formula: cf = {[migration in response to PAF or leukotriene B_4 (with or without drugs)]/[spontaneous migration]} − 1.

2.3. Measurement of cyclic AMP in eosinophils

After purification, eosinophils were resuspended in RPMI-1640 medium (pH = 7.2) containing 30 mM Hepes buffer and 2 mg/ml sodium bicarbonate. For measurement of cyclic AMP accumulation, 2 million cells were incubated with different drugs or respective vehicles. After centrifugation, cells were resuspended in 250 μl of ethanol and allowed to stand for 5 min before centrifugation to remove insoluble residues. The supernatant was collected and evaporated to dryness ~ 55°C under a stream of nitrogen. The residue was dissolved in 0.5 ml of 2-amino-2-hydroxymethylpropane-1,3-diol and ethylenediamine tetraacetic acid (Tris-EDTA) buffer. The cyclic AMP levels were determined using a cyclic AMP [^{3}H] assay system (Amersham International, UK), according to the instructions supplied by the manufacturer.

2.4. Platelet aggregation

Blood was collected from ether-anaesthetised Wistar rats by cardiac puncture, using sodium citrate (2%) as anticoagulant. Platelet-rich plasma was prepared by centrifugation at 200 × *g* for 15 min and platelet-poor plasma after centrifugation of the remaining blood at 2500 × *g* for 20 min. Final platelet concentration was adjusted to 600 million cells/ml. Platelet aggregation was determined at 37°C by means of a turbidimetric assay in a dual channel aggregometer (Chrono-log, USA). Adenosine 5′-diphosphate (ADP) at 10 μM was used as agonist. Phosphodiesterase type III and V inhibitors (0.001 and 0.1 μM) were incubated with platelet rich plasma for 2 min before the addition of ADP. Platelets pre-incubated with the respective vehicles were used as controls. Results were expressed as percentage of aggregation in relation to the response produced by ADP.

2.5. Materials

RPMI-1640 medium, Hepes, ovalbumin, leukotriene B_4, bovine serum albumin, Percoll, milrinone, Bt_2 cyclic AMP (*N*6-2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate), forskolin (7β-acetoxy-1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one), adenosine diphosphate (ADP) and zaprinast were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and PAF (1-*O*-hexadecyl-2-acetyl-*sn*-3-glycero-phosphorilcholine) from Novabiochem (Switzerland). Rolipram was a generous gift from the Institut de Recherche Jouveinal (France) and SK&F 94836 from Smith-Kline Beecham (Epsom, UK). Ro 20-1724 was obtained from RBI (Natick, MA, USA). Salbutamol and salmeterol were kindly provided by Dr. Fernando Vieira (Glaxo, Rio de Janeiro, Brazil). All solutions were freshly prepared immediately before use.

2.6. Statistical analysis

Data are reported as mean ± S.E.M. and were analysed by analysis of variance (ANOVA) followed by the Newman-Keuls-Student *t*-test. When comparing only two groups, the difference in means was analysed by unpaired Student's *t*-test. Probability values of 0.05 or less were considered significant.

3. Results

3.1. The chemotactic response of rat peritoneal eosinophils to PAF and leukotriene B_4

Consistent with previous observations, intact eosinophils showed a significant chemotactic response to PAF (Fig. 1A) or leukotriene B_4 (Fig. 1B), as measured in a modified Boyden chamber. From the concentrations tested for each agonist (0.001–10 μM), increasing the concentration gradient resulted in an elevated number of migrating eosinophils until a plateau was reached, after which the number of cells decreased. The optimal concentrations of PAF and leukotriene B_4 were of 1 μM and 0.1 μM, respectively, and these were selected for use in further experiments in order to test the influence of the cyclic AMP active agents on the eosinophil chemotaxis assay.

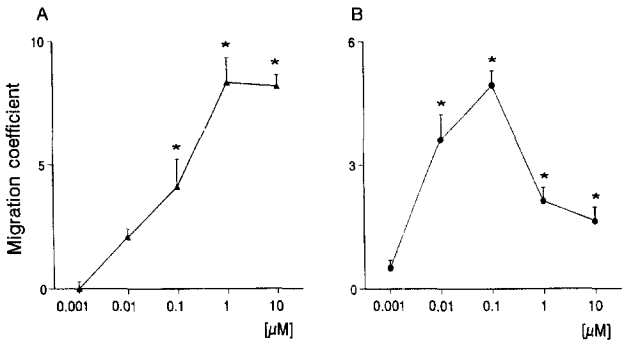

Fig. 1. Dose-response curve of PAF- (A) and leukotriene B_4- (B) induced eosinophil migration in the modified Boyden system. Each point represents the means ± S.E.M. of 5 separate experiments done in duplicate. * $P < 0.05$ as compared to spontaneous migration.

3.2. Effect of phosphodiesterase III, IV and V inhibitors on eosinophil chemotaxis

A 30-min incubation of the eosinophils with rolipram (0.1–100 μM), which is described as a phosphodiesterase IV selective inhibitor, produced a significant reduction of the PAF- (Fig. 2A) and leukotriene B_4-induced migration (Fig. 2B). Rolipram affected the chemotaxis induced by PAF more than the chemotaxis induced by leukotriene B_4. Another phosphodiesterase IV inhibitor, Ro 20-1724, exhibited significant inhibitory activity against leukotriene B_4-induced chemotaxis, like rolipram did. The chemotaxis index after treatment with 1, 10 or 100 μM of Ro 20-1724

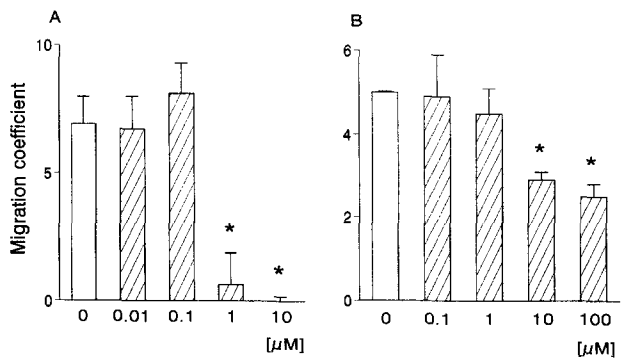

Fig. 2. Effect of rolipram (0.01–100 μM) on eosinophil chemotaxis induced by PAF (A) or leukotriene B_4 (B). Cells were pre-incubated with vehicle (open columns) or rolipram (hatched columns) for 30 min prior to stimulation with the chemoattractants. Data are expressed as means ± S.E.M. of 5 separate experiments done in duplicate. * $P < 0.05$ indicates statistical significance as compared to vehicle-treated group.

Table 1
Lack of effect of selective phosphodiesterase (PDE) III and V inhibitors on eosinophil chemotaxis induced by PAF or leukotriene (LT) B_4 in vitro

PDE inhibitor	Selectivity	[μM]	Chemotaxis index	
			LTB_4	PAF
Milrinone	Type III	0	n.d.	4.40 ± 0.60
		1	n.d.	4.80 ± 0.30
		10	n.d.	4.60 ± 0.10
SK&F 94836	Type III	0	11.1 ± 2.31	4.20 ± 0.30
		1	7.81 ± 0.99	4.70 ± 0.40
		10	8.80 ± 2.01	5.10 ± 0.30
Zaprinast	Type V	0	8.51 ± 3.93	4.67 ± 0.81
		1	6.43 ± 0.68	4.43 ± 0.96
		10	6.73 ± 1.17	5.19 ± 1.17

Cells were pre-incubated with the drugs for 30 min prior to stimulation with PAF (1 μM) or LTB_4 (0.1 μM). The range of random migration was 20–33 eosinophils/15 high-power fields. Data represent the means ± S.E.M. from 3–7 separate experiments done in duplicate.

was 4.8 ± 0.7, 2.5 ± 0.4 ($P < 0.05$) and 2.1 ± 0.4 ($P < 0.05$), respectively, as compared to the chemotaxis index of untreated control eosinophils of 5.0 ± 0.3. Experiments were performed to evaluate the effects of rolipram and Ro 20-1724 on intact eosinophil random motility. While spontaneous migration of untreated cells was 33.1 ± 3.5 eosinophils/15 high-power fields (mean ± S.E.M.), the migration in the presence of 1 or 100 μM of rolipram was 25.3 ± 1.2 or 27.9 ± 5.5 eosinophils/15 high-power fields (mean ± S.E.M.), respectively, and the migration in the presence of 1 or 100 μM of Ro 20-1724 was 29.5 ± 6.0 or 39.0 ± 7.0 eosinophils/15 high-power fields (mean ± S.E.M.), respectively. Cell viability was assessed in eosinophils after a 30 min incubation with rolipram or Ro 20-1724 (10 μM) by means of trypan blue extrusion. It appeared that rolipram and Ro 20-1724-treated eosinophils remained > 97% and > 99% viable. The phosphodiesterase III inhibitors milrinone and SK&F 94836 (1–100 μM) and the phosphodiesterase V inhibitor zaprinast (1–10 μM) did not modify the eosinophil chemotactic response to both chemoattractants (Table 1).

Table 2
Effect of selective phosphodiesterase III and V inhibitors on rat platelet aggregation induced by ADP in vitro

Inhibitor	Selectivity	n	[μM]	% of control
Milrinone	III	4	1	79.62 ± 11.26
		4	10	6.31 ± 6.21 [a]
SK&F 94836	III	4	1	62.47 ± 10.77
		5	10	8.23 ± 8.20 [a]
Zaprinast	V	3	10	161.03 ± 12.50 [a]
		3	100	196.04 ± 11.40 [a]

Platelets were pre-incubated with tested drugs for 2 min at 37°C. Control aggregation with ADP (10 μM) was 80.04 ± 11.40% ($n = 7$). Data represent the means ± S.E.M. from a number of independent experiments. [a] $P < 0.05$ indicates statistical significance as compared to vehicle-treated group.

Table 3
Failure of β_2-adrenoceptor agonists to inhibit eosinophil chemotaxis induced by PAF or leukotriene (LT) B_4 in vitro

Drugs	[μM]	n	PAF	LTB_4
Salbutamol	0	5	n.d.	5.06 ± 0.73
	1	6	n.d.	3.89 ± 0.19
	10	4	n.d.	6.17 ± 0.77
Salmeterol	0	6	9.12 ± 1.51	5.66 ± 0.65
	1	3	9.85 ± 1.56	3.02 ± 0.87
	10	3	8.78 ± 1.57	4.02 ± 0.77

Cells were pre-incubated with drugs or vehicle for 30 min prior to stimulation with PAF (1 μM) or LTB_4 (0.1 μM). The range of random migration was 20–33 eosinophils/15 high-power fields. Data represent the means ± S.E.M. from 4–6 separate experiments done in duplicate.

3.3. Effect of phosphodiesterase III and V inhibitors on platelet aggregation

To ascertain the pharmacological activity of the phosphodiesterase III and V inhibitors, we used the in vitro model of platelet aggregation based on the turbidimetric principle. Milrinone and SK&F 94836 (1–10 μM) exhibited important inhibitory activity on ADP-induced rat platelet aggregation (maximum inhibition of 91.8 ± 8.2%, $P < 0.001$, and 93.7 ± 6.3%, $P < 0.001$, respectively), while zaprinast (10–100 μM) increased the platelet response to ADP (10 μM) (61.0 ± 12.5% and 96.6% ± 11.4%, respectively, $P < 0.001$) (Table 2).

3.4. Effect of β_2-adrenoceptor agonists, forskolin and Bt_2 cyclic AMP on eosinophil chemotaxis

We further examined the effect of adenylate cyclase activation either by indirect stimulation with short- and

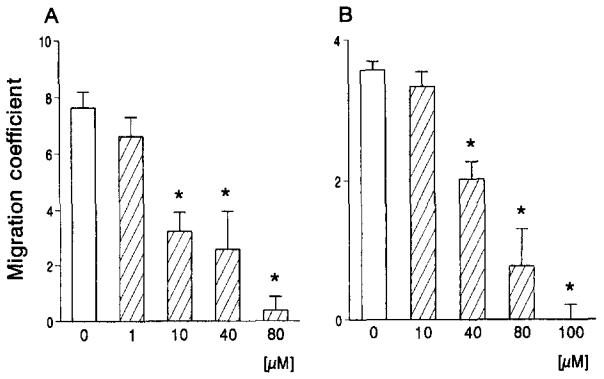

Fig. 3. Inhibition by forskolin (1–100 μM) of eosinophil chemotaxis induced by PAF (1 μM) (A) or leukotriene B_4 (0.1 μM) (B). Cells were pre-incubated with vehicle (open columns) or forskolin (hatched columns) for 30 min before exposure to chemoattractants. The data are expressed as means ± S.E.M. of 4 separate experiments done in duplicate. * $P < 0.05$ indicates statistical significance as compared to vehicle-treated group.

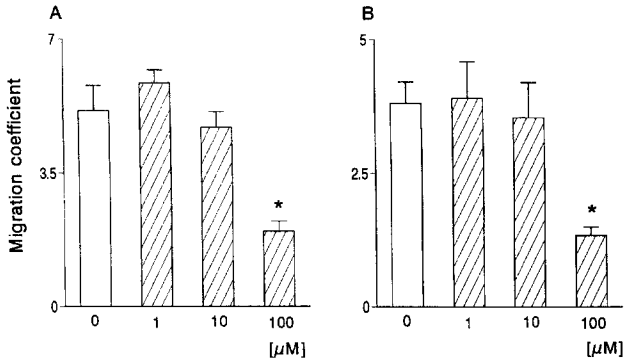

Fig. 4. Effect of Bt_2 cyclic AMP (1–100 μM) on eosinophil chemotaxis induced by PAF (1 μM) (A) or leukotriene B_4 (0.1 μM) (B). Cells were pre-incubated with vehicle (open columns) or Bt_2 cyclic AMP (hatched columns) for 30 min prior to stimulation with chemoattractants. The data are expressed as means ± S.E.M. of 4 separate experiments done in duplicate. * $P < 0.05$ indicates statistical significance as compared to vehicle-treated group.

long-acting β_2-adrenoceptor agonists salbutamol and salmeterol, respectively, or by direct stimulation with forskolin. Salbutamol and salmeterol, at concentrations ranging from 1 to 100 μM, were ineffective in inhibiting the chemotaxis response induced by PAF or leukotriene B_4 (Table 3). In contrast, forskolin (10–100 μM) caused a concentration-dependent reduction in the number of migrating eosinophils stimulated by PAF (Fig. 3A) or leukotriene B_4 (Fig. 3B), though the chemotaxis elicited by the former was more sensitive than the chemotaxis to the latter. Further experiments were performed to determine the effect of the analogue of cyclic AMP, Bt_2 cyclic AMP, on this migration system. Eosinophil chemotaxis in response to PAF or leukotriene B_4 was inhibited by Bt_2 cyclic AMP, though only the highest concentration (100 μM) altered significantly the chemotactic response (Fig. 4A and B, respectively). Exposure of eosinophils to forskolin in the presence of rolipram, at concentrations which alone had no effect, produced a significant reduction

Table 4
Effect of isoenzyme-selective phosphodiesterase inhibitors, forskolin and β_2-adrenoceptor agonists on eosinophil cyclic AMP accumulation

Drug	[μM]	n	Cyclic AMP (pmol/2×10^6 cells)
None	–	4	1.20±0.01
Rolipram	10	3	2.68±0.69 [a]
SK&F 94836	100	4	1.27±0.03
Forskolin	10	3	1.76±0.18 [a]
Salbutamol	10	3	1.26±0.04
Salmeterol	10	3	1.27±0.05
Rolipram/forskolin	0.1/1	3	1.25±0.03

Cells were pre-incubated with tested drugs for 30 min at 37°C. Concentrations of cyclic AMP were assayed as described in Materials and methods. Data represent the means ± S.E.M. from 3–4 independent experiments done in duplicate. [a] $P < 0.05$ indicates statistical significance as compared to control group.

of the PAF- (Fig. 5A) and leukotriene B_4-induced eosinophil migration (Fig. 5B).

3.5. Effects of phosphodiesterase inhibitors, β_2-adrenoceptor agonists and forskolin on cyclic AMP levels of eosinophils

In order to establish an association between the suppression of eosinophil chemotaxis and the ability of the agents tested to increase intracellular cyclic AMP further, we measured cyclic AMP levels in intact eosinophils. Table 4 shows that a 30-min incubation of cells with the phosphodiesterase IV inhibitor rolipram elicited a significant increase of cyclic AMP levels, to about 2-fold those of control samples ($P < 0.05$), showing a good correlation with its capacity to inhibit eosinophil chemotaxis. Though to lesser extent, forskolin at the concentration of 10 μM also significantly elevated cyclic AMP levels (1.5-fold, $P < 0.05$). Paradoxically, incubation of cells with the combination of rolipram and forskolin did not modify the intracellular levels of cyclic AMP, conditions under which

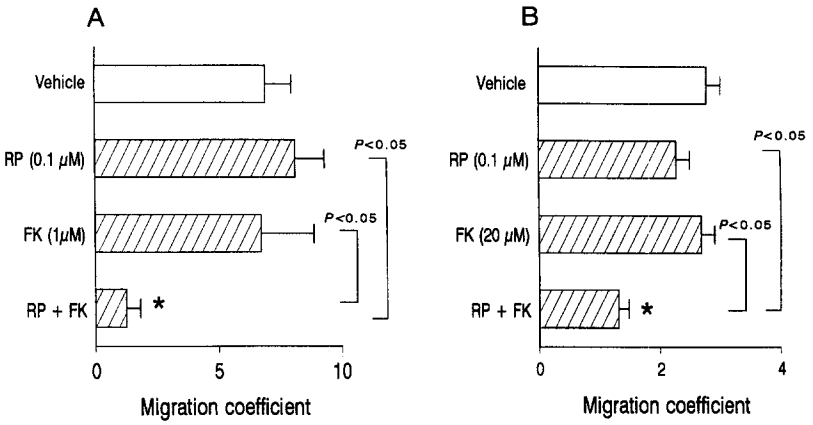

Fig. 5. Inhibitory effect of forskolin on PAF (1 μM)- (A) or leukotriene B_4 (0.1 μM)- (B) induced eosinophil chemotaxis in the presence of rolipram. Forskolin and rolipram were incubated alone or in combination (hatched columns) for 30 min prior to stimulation with chemoattractants. Open columns represent the migration observed after incubation of eosinophils with vehicle. The data are expressed as means ± S.E.M. of 4 separate experiments done in duplicate.

the migratory response was abolished. In contrast, consistent with the lack of effect on eosinophil migration, no significant change in cyclic AMP levels was observed with the phosphodiesterase III inhibitor SK&F 94836 (100 μM) or β_2-adrenoceptor agonists salbutamol and salmeterol when tested at the concentration of 10 μM.

4. Discussion

The present study demonstrates that type IV isoenzyme phosphodiesterase inhibitors directly reduce rat eosinophil chemotaxis in vitro, and suggest that this effect may be dissociated from the recognized ability of these drugs to elevate eosinophil cyclic AMP. We found that the phosphodiesterase IV inhibitor rolipram impaired the eosinophil chemotactic response to PAF or leukotriene B_4, in association with a rise in cyclic AMP intracellular levels, whereas the type III phosphodiesterase inhibitors milrinone and SK&F 94836 and the phosphodiesterase V inhibitor zaprinast were inactive. Forskolin was shown to be a potent inhibitor of eosinophil chemotaxis although it produced only a weak elevation of intracellular cyclic AMP levels. Moreover, the association of ineffective doses of forskolin and rolipram synergistically inhibited the eosinophil response without changing the cyclic AMP concentration.

Eosinophils appear to be recruited into sites undergoing hypersensitivity reactions or immunologically mediated diseases (Gleich et al., 1987; Kroegel et al., 1994) by a mechanism at least partially dependent on cell adhesion and chemotaxis (Walsh et al., 1993). Thus, inhibition of eosinophil infiltration into the inflammatory focus is believed to be an important strategy to control allergic diseases. Specific and non-specific phosphodiesterase inhibitors have been shown to prevent allergen-induced tissue eosinophil accumulation in different animal species including guinea-pigs (Newsholme and Schwartz, 1993; Lagente et al., 1994; Santing et al., 1995), rats (Elwood et al., 1995), mice (Nagai et al., 1995) and humans (Djukanovic et al., 1995). Previous studies have demonstrated that phosphodiesterase type IV is responsible for cyclic AMP catabolism in eosinophils (Torphy and Undem, 1991; Dent et al., 1991) as well as in other cells including macrophages (Turner et al., 1993), lymphocytes (Robicsek et al., 1991) and neutrophils (Plaut et al., 1983). Also via breakdown of cyclic AMP, phosphodiesterase III is required in the regulation of airway smooth muscle tone, whereas phosphodiesterase V is involved in the breakdown of cyclic GMP in the airway and vascular smooth muscle (Barnes, 1995).

In the present study, the direct effect of selective phosphodiesterase IV inhibitors on rat eosinophil chemotaxis triggered by PAF or leukotriene B_4 was investigated. Phosphodiesterase III and V selective inhibitors and activators of cyclic AMP pathway were also tested for comparison. For that purpose we used the modified Boyden chamber system and peritoneal eosinophils, which were purified (about 90%) from naive Wistar rats by a Percoll separation technique. It is noteworthy that the eosinophils were obtained from the peritoneal cavity of non-stimulated rats; under normal conditions the number of eosinophils is about 15–20% of the number of free recovered leucocytes (Martins et al., 1989). This is a relevant strategy because eosinophils are usually taken from eosinophilic patients with allergy or parasitic diseases, or from serum- or polymyxin-treated animals (Shute, 1993). As previously demonstrated, these cells are clearly in an activated condition, since they were recruited into the inflammatory site (Mengelers et al., 1994; Sedgwick et al., 1992). Both PAF and leukotriene B_4 (0.001–10 μM) yielded concentration-dependent migratory responses which peaked at 1 μM and 0.1 μM, respectively. The higher chemotactic activity expressed by the latter is consistent with that of previous studies, which have shown that eosinophils from human and guinea-pig are also more sensitive to leukotriene B_4 than to PAF on a molar basis. Our results showed that pre-incubation with the phosphodiesterase IV inhibitor rolipram (0.001–10 μM) dose dependently abolished the response to PAF ($IC_{50} = 0.73$ μM), while the maximal suppression of leukotriene B_4-induced eosinophil chemotaxis was approximately 60% ($IC_{50} = 48$ μM). Ro 20-1724, another phosphodiesterase IV inhibitor, impaired the chemotactic response to leukotriene B_4 with a similar profile (IC_{50} of 28 μM). The distinct potency and efficacy with which rolipram affects eosinophil chemotaxis triggered by either PAF or leukotriene B_4 suggests that the impact of phosphodiesterase IV blockade may depend on the type of chemoattractant receptor activated. From other studies, including eosinophil secretion, it became evident that the potency of compounds able to inhibit eosinophil functions indeed depends on the stimulus applied (Kita et al., 1991). Since cyclic AMP-protein kinase A activation is recognized to uncouple heterologous receptors from phosphoinositide-specific phospholipase C, differences in the receptor reserve and/or post-receptor signal transduction mechanisms may determine the differential sensitivity of PAF- and leukotriene B_4-evoked responses to the phosphodiesterase IV blockade.

We have found that inhibitors of the other isoenzymes milrinone and SK&F 94836 (type III) and zaprinast (type V) failed to modify eosinophil migration in response to PAF and leukotriene B_4. The present data are consistent with previous observations that inhibitors of phosphodiesterase III and V are ineffective against some eosinophil functions including respiratory burst and secretion of granules constituents (Dent et al., 1991; Hatzelmann et al., 1995). In addition, treatment with such drugs did not alter the eosinophil accumulation in vivo noted in models of antigen-induced guinea-pig airway inflammation (Lagente et al., 1994) and rat pleurisy (in preparation). In another set of experiments, rat platelets were used in order to confirm the pharmacological activity of our samples of milrinone,

SK&F 94836 and zaprinast. Indeed, at the same concentrations used in the chemotaxis assay, all these drugs were able to interfere with platelet aggregation. Incubation of platelets with milrinone and SK&F 94836 markedly attenuated the aggregation induced by ADP in vitro. Zaprinast produced a considerable enhancement of platelet aggregation, an effect which might be possibly dependent on its capacity to elevate intracellular levels of cyclic GMP. In fact, incubation of platelets with the analogue Bt_2 cyclic GMP did produce a similar positive modulatory effect on rat platelets (data not shown).

The modulatory effect of phosphodiesterase IV inhibitors is supposed to be associated with elevation of the intracellular levels of cyclic AMP as a consequence of impairment of its degradation (Hall, 1993). In fact, we found that incubation of peritoneal eosinophils with 10 μM rolipram led to a 2.6-fold increase in the intracellular levels of cyclic AMP as compared to those of vehicle-treated cells, whereas the phosphodiesterase type III inhibitor SK&F 94836 had no effect. Using alternative approaches to increase cyclic AMP concentrations in eosinophils, we tested the effects of compounds able to activate adenylate cyclase such as forskolin and β_2-adrenoceptor agonists. We showed that salbutamol and salmeterol did not modify cyclic AMP baseline levels and also failed to inhibit eosinophil chemotaxis in vitro. Though the effectiveness of β_2-adrenoceptor agonists on eosinophil functions is somewhat controversial, our findings indicate that the lack of effect of such drugs under our conditions may possibly depend on a structural alteration, functional deficiency and/or the absence or lower density of β_2-adrenoceptors on rat eosinophils (Yukawa et al., 1990; Muñoz et al., 1995). By contrast, salbutamol suppressed antigen-induced pleural eosinophil accumulation in rats, suggesting that its inhibitory effect in vivo is not dependent on a direct action on the migratory capacity of eosinophils (Diaz et al., 1996). While producing a significant but very weak stimulatory effect on cyclic AMP levels, forskolin was able to abolish PAF- and leukotriene B_4-induced eosinophil chemotaxis, showing IC_{50} values of 10 μM and 42 μM, respectively. As observed for rolipram, PAF-evoked eosinophil migration appears to be more sensitive to forskolin than is leukotriene B_4-evoked migration. Moreover, the synergistic inhibitory effect provided by the combined forskolin plus rolipram treatment was also more prominent following eosinophil activaction by PAF than after activation by leukotriene B_4. Our findings are in line with those of Harvath et al. (1991), who demonstrated that forskolin differentially affected neutrophil responsiveness, inhibiting FMLP- but not leukotriene B_4-induced chemotaxis. All together, these results support the interpretation that the control exerted by cyclic AMP modulators upon polymorphonuclear leucocyte chemotaxis is clearly dependent on the stimulatory signal. It is of interest to note that the synergistic inhibition observed after the combined forskolin plus rolipram treatment was not followed by elevation of eosinophil cyclic AMP. This result suggests a dissociation between the inhibitory effect noted after adenylate cyclase activation and phosphodiesterase IV inhibition and changes in the intracellular levels of cyclic AMP, as has been previously reported for other biological systems. The fact that eosinophil chemotaxis elicited by either PAF or leukotriene B_4 was at best inhibited to only 60% after a high concentration (1000 μM) of the non-hydrolysable analogue Bt_2 cyclic AMP adds support to this interpretation. Nevertheless, it is possible that the slight or no increase of eosinophil cyclic AMP levels noted in our experiments may depend on the very rapid and transient nature of this phenomenon and/or on the considerable amount of the nucleotide that is extruded into the extracellular environment (Harvath et al., 1991). Alternatively, a more significant increase of cyclic AMP concentrations may take place in some particular compartments where the enzymes responsible for the migratory response are localized (Vegesna and Diamond, 1984). This does not necessarily result in an increase in the total amount of cellular cyclic AMP. And there is also the possibility that cyclic AMP-stimulator compounds inhibited eosinophil chemotaxis by additional mechanisms including blockade of extracellular calcium mobilization (Botana et al., 1994), direct activation of AMP-dependent protein kinase (Souness et al., 1991) and/or endogenous production of adenosine (Derian et al., 1994).

The clinical relevance of phosphodiesterase isoenzyme inhibition in asthma is still unknown. However, Sullivan et al. (1994) demonstrated that atopic asthmatic patients chronically treated with low doses of the phosphodiesterase inhibitor theophylline showed a reduction in the number of activated eosinophils present in the bronchial wall following antigen stimulation. In this study, we show that eosinophil chemotaxis can be prevented by a direct effect of phosphodiesterase IV inhibitors, whereas phosphodiesterase III or V selective blockers are inactive. These findings further indicate that the anti-migratory effect of phosphodiesterase IV inhibitors may explain the efficacy of these drugs in inhibiting tissue eosinophil accumulation.

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