Effect of Selective Phosphodiesterase Inhibitors on the Rat Eosinophil Chemotactic Response *In Vitro*

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In the present study, we have performed a comparative analysis of the effect of selective inhibitors of phosphodiesterase (PDE) type III, IV and V on eosinophil chemotaxis triggered by platelet activating factor (PAF) and leukotriene B_4 (LTB_4) in vitro*. The effect of the analogues N6-2'-O-dibutyryladenosine 3':5' cyclic monophosphate (Bt_2 cyclic AMP) and N2-2'-O- dibutyrylguanosine 3':5' cyclic monophosphate (Bt_2 cyclic GMP) has also been determined. The eosinophils were obtained from the peritoneal cavity of naive Wistar rats and purified in discontinuous Percoll gradients to 85-95% purity. We observed that pre-incubation of eosinophils with the PDE type IV inhibitor rolipram suppressed the chemotactic response triggered by PAF and LTB_4, in association with an increase in the intracellular levels of cyclic AMP. In contrast, neither zaprinast (type V inhibitor) nor type III inhibitors milrinone and SK&F 94836 affected the eosinophil migration. Only at the highest concentration tested did the analogue Bt_2 cyclic AMP suppress the eosinophil chemotaxis, under conditions where Bt_2 cyclic GMP was ineffective. We have concluded that inhibition of PDE IV, but not PDE III or V, was able to block the eosinophil chemotaxis* in vitro*, suggesting that the suppressive activity of selective PDE IV inhibitors on tissue eosinophil accumulation may, at least, be partially dependent on their ability to directly inhibit the eosinophil migration.*

Key words: eosinophil - migration - phosphodiesterase inhibitors

The cyclic nucleotides are considered important second messengers in a wide variety of biological systems due to their regulatory role in the cell activity (Kaliner & Austen 1974). The control of the intracellular levels of cyclic nucleotides has been shown to be mainly dependent on their breakdown by phosphodiesterases (PDE) (Beavo et al. 1994), which now comprises at least seven families of distinct isoenzymes (Conti et al. 1995). A variety of selective inhibitors of PDE are available, permitting the pharmacological manipulation of different physiopathological processes (Barnes 1995). Eosinophils are believed to play a relevant role in allergy and their accumulation in several tissues is considered an important feature of hypersensitivity reactions or immunologically mediated diseases (Kroegel et al. 1994). Treatment with non-selective or selective PDE IV inhibitors has been shown effective in inhibiting eosinophil recruitment after stimulation with antigen, PAF or IL-5 (Barnes & Pauwels 1994). In this study, we have examined the ability of selective inhibitors of PDE IV as well as those of PDE III and V to inhibit the eosinophil chemotaxis in a modified Boyden chamber. The effect of the cell permeable analogues Bt_2 cyclic AMP and Bt_2 cyclic GMP has been also evaluated.

MATERIALS AND METHODS

Eosinophils were obtained from the peritoneal cavity of ether-anaesthetised Wistar rats and the purification was performed using Percoll density gradients (72% and 56% solutions). The number of cells was counted in the Neubauer chamber and the differential analysis performed in cytospin preparations stained with May-Grunwald-Giemsa dye. Cell viability was evaluated by trypan blue exclusion and eosinophils of 85-95% purity and 96% viability were used in the following experiments. For the migration experiments, 48-well microchemotaxis chamber and cellulose nitrate filters (3 µm pore) (Neuro Probe, Inc., USA) were used in accordance with the technique described by Richards and McCullough (1984). Leukotriene

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B_4 (LTB_4) (Sigma Chemical Co, St. Louis, MO) and platelet-activating factor (PAF) (Novabiochem, Switzerland) were used as chemoattractants. To test the interference of PDE inhibitors or cyclic nucleotide analogues, purified eosinophils were pre-incubated with either drug or with their respective vehicle at 37°C for 30 min, in a 5% CO_2: 95% O_2 atmosphere. Rolipram (a generous gift from the Institut de Recherche Jouveinal, France), SK&F 94836 (Smith-Kline Beecham, U.K.), milrinone and zaprinast (Sigma Chemical Co, St. Louis, MO) were dissolved in 20% Tween 80 and diluted to the desired concentration with saline. Bt_2 cyclic AMP and Bt_2 cyclic GMP (Sigma Chemical Co, St. Louis, MO) were dissolved in saline solution. The chemotaxis chamber was incubated for 2 hr at 37°C in a 5% CO_2:95% O_2 atmosphere and the filter fixed and stained as described. Eosinophils migrated at 40 µm from the upper surface of the filter were counted in 15 high-power fields (hpf) and the results expressed as a coefficient migration (cf), calculated according to the formula:

$$cf = \frac{\text{migration in response to PAF or } LTB_4 \text{ (with or without drugs)}}{\text{spontaneous migration}} - 1$$

The cyclic AMP levels were determined using a cyclic AMP [^{3}H] assay system (Amersham International plc, UK), according to the instructions supplied by the manufacturer. Data are reported as means ± S.E.M. and were analysed by analysis of variance (ANOVA), followed by the Newman-Keuls-Student's t test and values of 0.05 or less were considered significant.

RESULTS

Consistent with our previous observations, eosinophils presented a significant chemotactic response to PAF or LTB_4, with optimal concentrations of 1 µM and 0.1 µM respectively (Martins et al. 1988). We noted that pre-incubation of the eosinophils with the PDE IV inhibitor rolipram (1-100 µM) abrogated PAF- and LTB_4-induced chemotaxis (Table I). Similarly, treatment of eosinophils with RO 20-1724, another PDE IV inhibitor, also suppressed the eosinophil migration to LTB_4. Chemotaxis index of 4.8 ± 0.7 and 2.1 ± 0.4 was observed after treatment with 1 and 100 µM of the RO 20-1724, respectively, when compared to that of 5.0 ± 0.3 for untreated eosinophils. It is important to note that no alteration of the random motility of eosinophils was noted after rolipram or RO 20-1724. Values of spontaneous migration of untreated cells were 33.1 ± 3.5 eosinophils/15 hpf (mean ± S.E.M.) and the migration in the presence of 100 µM of rolipram or RO 20-1724 was 27.9 ± 5.5 and 39.0 ± 7.0 eosinophils/15 hpf respectively. Eosinophils treated with rolipram and Ro 20-1724 remained >97% and >99% viable as attested by the trypan blue exclusion. In contrast, milrinone and SK&F 94836 (type III inhibitors) (1-100 µM) and zaprinast (type V inhibitor) (1-100 µM) did not modify the eosinophil chemotactic response to both chemoattractants (Table I). We further examined the effect of the cyclic nucleotide analogues on the chemotaxis system. Our data indicate that Bt_2 cyclic AMP, though only at the highest concentration (100 µM), inhibited PAF- and LTB_4-induced eosinophil chemotaxis, whereas Bt_2 cyclic GMP was ineffective (Fig.). To verify the relationship between intracellular cyclic AMP and inhibition of eosinophil migration, we measured the levels of this nucleotide in eosinophils. Incubation for 30 min with PDE IV inhibitor rolipram led to a 2-fold increase in the intracellular levels of cyclic AMP as compared to control samples (Table II). No significant alteration in cyclic AMP levels was observed with the PDE III inhibitor SK&F 94836 (100 µM).

TABLE I

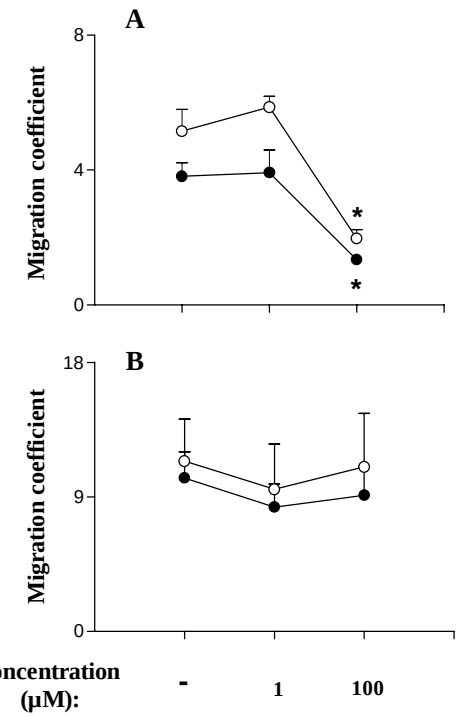
Effect of selective PDE III, IV and V inhibitors on eosinophil chemotaxis induced by PAF or LTB_4 *in vitro*

Cyclic AMP modulators	[µM]	Migration coefficient PAF	Migration coefficient LTB_4
None	-	7.07 ± 2.07	4.56 ± 0.28
Rolipram	1	1.24 ± 1.07[a]	4.50 ± 0.60
	10	0.01 ± 0.17[a]	2.90 ± 0.20[a]
	100	n.d.	2.50 ± 0.30[a]
SK&F 94836	1	7.71 ± 0.99	4.70 ± 0.40
	10	8.80 ± 2.01	5.10 ± 0.30
	100	n.d.	3.90 ± 0.30
Zaprinast	1	6.43 ± 0.68	4.43 ± 0.96
	10	6.73 ± 0.86	5.19 ± 1.17
	100	n.d.	3.60 ± 0.93

Cells were pre-incubated with the tested drugs for 30 min, at 37°C, before stimulation with PAF (1 µM) or LTB_4 (0.1 µM). Data represent the mean ± SEM from a number of 3-7 experiments made in duplicate. [a]*P<0.05* indicates statistical significance as compared to control group.

DISCUSSION

Specific and non-specific PDE inhibitors were shown to abolish allergen-induced tissue eosinophil accumulation in different animal species (Lagente et al. 1994, Elwood et al. 1995, Djukanovic et al. 1995). Our results showed that pre-incubation of eosinophils with the PDE IV inhibitor rolipram (1 to 100 µM) dose-dependently inhibited the response to PAF (IC_{50} = 0.73 µM),



Effect of Bt_2 cyclic AMP (A) and Bt_2 cyclic GMP (B) on the eosinophil chemotaxis induced by PAF (1 μM) (open circles) or LTB_4 (0.1 μM) (closed circles). Eosinophils were treated with drugs or vehicles for 30 min. The results are expressed as means ± S.E.M. of 4-6 independent experiments done in duplicate. * $p<0.05$ indicates statistical significance as compared to control group.

TABLE II

Effect of selective PDE inhibitors on eosinophil cyclic AMP accumulation

Drugs	[μM]	n	Cyclic AMP (pmol/ $2x10^6$ cells)
None	-	4	1.20 ± 0.01
Rolipram	10	3	2.68 ± 0.69[a]
SK&F 94836	100	4	1.27 ± 0.03

Cells were pre-incubated with tested drugs for 30 min at 37ºC. 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.

while the maximal suppression of LTB_4-induced eosinophil chemotaxis was approximately 60% (IC_{50} = 48 μM). Likewise, another PDE IV inhibitor RO 20-1724 suppressed the chemotactic response to LTB_4 in the same way as rolipram. The distinct potency and efficacy with which rolipram affected eosinophil migration evoked by either PAF or LTB_4 suggested that the impact of PDE IV blockade clearly depended on the type of chemoattractant receptor activated (Kita et al. 1991). Distinction in the receptor reserve and/or post-receptor signal transduction mechanisms may lead to differential sensitivity of PAF- and LTB_4-evoked responses to the PDE IV blockade. We also found that milrinone and SK&F 94836 (type lll inhibitors) and zaprinast (type V inhibitor) had no effect on eosinophil migration caused by both chemoattractants. At the same concentrations used in the chemotaxis assay, milrinone and SK&F 94836 inhibited platelet aggregation triggered by ADP, whereas zaprinast enhanced the phenomenon (Alves et al. 1996). These findings confirmed that our samples of PDE III and V inhibitors were pharmacological active, and reinforced that the prominent isoenzyme in eosinophils is PDE IV (Barnette et al. 1995). In another set of experiments, we demonstrated that incubation of eosinophils with rolipram raised cyclic AMP 2.6-fold above the basal levels detected in the vehicle-treated cells. The fact that 10 μM rolipram produced a modest increase in cyclic AMP levels, while abolishing the eosinophil chemotaxis, suggests the dissociation between PDE IV inhibition and changes in the intracellular levels of cyclic AMP. Nevertheless, it should be considered that the slight increase in the eosinophil cyclic AMP may be dependent on (i) the rapid/transient nature of cyclic AMP level changes, (ii) its extrusion to the extracellular environment and (iii) compartimentalization of cyclic nucleotide alterations (Vegesna & Diamond, 1984). Alternatively, rolipram may possibly be inhibiting the eosinophil chemotactic response by mechanisms other than only PDE IV inhibition. Consistent with the lack of activity in eosinophil migration, no significant changes in cyclic AMP levels were noted with SK&F 94836 (100 μM). Considering the effect of the cell permeable cyclic nucleotide analogues, we observed that either PAF or LTB_4 was at best inhibited to only 60% after high concentration (100 μM), supporting the idea that there is not always a direct association between the suppression of biological responses and elevation of cyclic AMP levels. Bt_2 cyclic GMP showed itself quite ineffective in blocking eosinophil chemotaxis.

In conclusion, our results showed that eosinophil chemotaxis can be abrogated by selective PDE IV inhibitors, but not those of PDE III or V, by a mechanism dependent on the type of chemoattractant receptor activated. In addition, they also indicated that the effectiveness of PDE IV inhibitors to abolish tissue eosinophil accumulation may be accounted for by their anti-migratory activity.

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