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Effects of agents which elevate cyclic AMP on guinea-pig eosinophil homotypic aggregation

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1 Eosinophil recruitment and activation in inflamed tissue is thought to play an important role in the pathophysiology of allergic diseases. Experimental evidence suggests that elevating cyclic AMP is an effective means of reducing eosinophil recruitment *in vivo* and may therefore have therapeutic benefit. In the present study, we have assessed the capacity of cyclic AMP-elevating agents to modulate guinea-pig eosinophil homotypic aggregation, a CD18-dependent process, which may be an important component of eosinophil function *in vivo*.

2 Prostaglandin E_1 (PGE_1, 10^{-10} to 10^{-6} M) inhibited platelet activating-factor (PAF)- and C5a-induced eosinophil aggregation in a concentration-dependent manner. However, PAF-induced responses were more potently and more effectively inhibited by PGE_1. The inhibitory effects of PGE_1 on PAF-induced aggregation were reversed by pretreatment of eosinophils with the protein kinase A inhibitors H89 and KT5720.

3 The β_2-adrenoceptor agonists, salbutamol and salmeterol, concentration-dependently inhibited eosinophil aggregation induced by C5a and PAF and, again, PAF-induced responses were more effectively reduced. The inhibitory effect of salmeterol was mediated by β-adrenoceptors, as assessed by the reversal after pretreatment with propranolol.

4 Rolipram, a selective phosphodiesterase 4 (PDE4) inhibitor, also attenuated PAF- and C5a-induced aggregation and at a low concentration which did not affect aggregation *per se*, had a synergistic effect with PGE_1 and salbutamol to suppress this response.

5 Activation of eosinophils with PAF or C5a induced a small but significant increase in the level of CD18 expression on the eosinophil surface. PGE_1 (10^{-7} M) decreased PAF- and C5a-induced upregulation of CD18 by 93% and 62%, respectively.

6 These results demonstrate that cyclic AMP-elevating agents effectively inhibit eosinophil aggregation, a CD18-dependent functional response. Because CD18 has been shown to be important for eosinophil recruitment to inflamed tissue *in vivo*, our findings may be of relevance to the efficacy of cyclic AMP-elevating agents at inhibiting eosinophil trafficking.

Keywords: Eosinophils; CD18; cyclic AMP; phosphodiesterase inhibitors; β_2-adrenoceptor agonists; prostaglandins; aggregation

Introduction

Eosinophils are thought to play an important role in the pathophysiology of allergic diseases such as asthma and atopic dermatitis (Butterfield & Leiferman, 1993). In these conditions, eosinophil numbers and eosinophil-derived secretory products (eg. eosinophil major basic protein) are elevated in inflamed tissue and appear to correlate positively with the severity of the diseases (Djukanovic *et al.*, 1990; Gleich *et al.*, 1993). In addition, the activation status of eosinophils, as assessed by monoclonal antibodies such as EG2 (which recognizes the secreted form of eosinophil cationic protein), also correlates with functional indices of diseases severity (Djukanovic *et al.*, 1990; Corrigan & Kay, 1992). Inasmuch as the secretory products of eosinophils may cause tissue damage (eg. to epithelial cells and nerves) in concentrations which are found *in vivo* (Djukanovic *et al.*, 1990), the development of drugs which inhibit eosinophil recruitment and activation in the tissues may be of therapeutic value in the treatment of allergic diseases.

Recently, there has been renewed interest in a family of enzymes, collectively known as cyclic nucleotide phosphodiesterases (PDEs), which metabolize adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-guanosine monophosphate (cyclic GMP). It is now appreciated that PDEs are a diverse group of enzymes of which at least seven different families have been described (see Giembycz & Kelly, 1994). Of special interest is the finding that most cells implicated in the pathogenesis of inflammation express one or more representatives of the PDE4 isoenzyme family which are primarily or even exclusively responsible for the degradation of cyclic AMP in these cells (see Torphy & Undem, 1991; Giembycz, 1992). Accordingly, PDE4 inhibitors are capable of increasing cyclic AMP levels and inhibiting various functional responses (eg. respiratory burst) in most leukocytes that are considered pro-inflammatory (reviewed in Torphy & Undem, 1991; Giembycz, 1992). In addition, there is increasing evidence that PDE4 inhibitors suppress leukocyte recruitment, and specifically eosinophil recruitment, *in vivo* (eg. Howel *et al.*, 1993; Teixeira *et al.*, 1994b; Underwood *et al.*, 1993; 1994). Indeed, we have previously shown that the PDE4 inhibitor rolipram is effective at inhibiting the recruitment of eosinophils into sites of acute inflammation in guinea-pig skin at a dose which had no effect on neutrophil recruitment or oedema formation (Teixeira *et al.*, 1994b). Moreover, cyclic AMP-elevating agents including prostaglandin (PGE_1) and salbutamol also effectively inhibit eosinophil recruitment to sites of inflammation *in vivo* (Ting *et al.*, 1983; Fugner, 1989; Whelan & Johnson, 1992; Teixeira *et al.*, 1993; 1995b). Thus, there is considerable experimental evidence to suggest that cyclic AMP elevating agents are effective inhibitors of eosinophil recruitment *in vivo*. Despite these data, the precise cellular target for the inhibitory action of these drugs in such complex *in vivo* systems is unknown.

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The effects of cyclic AMP-elevating agents on eosinophil function *in vitro* have also been evaluated. It is clear from these studies that PDE4 inhibitors, β_2-adrenoceptor agonists and E-series prostaglandins inhibit several aspects of eosinophil function, including the respiratory burst (Souness *et al.*, 1991; Dent *et al.*, 1991; 1995; Barnette *et al.*, 1995), degranulation (Kita *et al*, 1991; Munoz *et al.*, 1994; Hatzelmann *et al.*, 1995; Souness *et al.*, 1995) and lipid mediator production (Munoz *et al.*, 1994; Souness *et al.*, 1994). However, we are not aware of any study evaluating the effects of cyclic AMP elevating agents on a CD18-dependent functional response of eosinophils *in vitro*. This is particularly relevant because CD18 is important for eosinophil recruitment *in vivo* (Milne & Piper, 1994; Teixeira *et al.*, 1994a; Das *et al.*, 1995). *In vitro*, after stimulation with various agonists, eosinophils undergo a time- and concentration-dependent homotypic aggregation (Teixeira *et al.*, 1995a). This response is calcium- and magnesium-dependent and relies largely on CD18 present on the eosinophil surface (Teixeira *et al.*, 1995a; 1996). In this study, we have investigated the ability of a range of cyclic AMP-elevating agents (E-series prostaglandins, β_2-adrenoceptor agonists and a PDE4 inhibitor) to interfere with guinea-pig eosinophil homotypic aggregation. Aggregation was assessed by changes in light transmission after activation of these cells with PAF and the complement fragment C5a.

Methods

Purification of guinea-pig peritoneal eosinophils

Eosinophils were harvested and purified as detailed elsewhere (Teixeira *et al.*, 1993; 1994b). Briefly, ex-breeder female guinea-pigs (Harlan, Oxon; 700–800 g) were treated with undiluted horse serum (1 ml i.p.) every other day for two to three weeks and the cells collected by peritoneal lavage with heparinized saline (10 iu ml^{-1}) 2 days after the last injection. The cells obtained were layered onto a discontinuous Percoll-HBSS (calcium- and magnesium-free) gradient followed by centrifugation (1500 × *g*, 25 min at 20°C). Eosinophils (>95% pure, >98% viable) were collected from the 1.090/1.095 and 1.095/1.100 g ml^{-1} density interfaces. The cells were then washed twice in phosphate buffered saline (PBS, calcium- and magnesium-free, pH 7.4) to which glucose (10 mM), $CaCl_2$ and $MgCl_2$ (final concentrations 1.0 mM and 0.7 mM, respectively) were added, and the cells kept on ice. Ten minutes before use, the cells were warmed to 37°C.

Eosinophil aggregation

Aggregation experiments were carried out as previously described (Teixeira *et al.*, 1995a; 1996). Briefly, guinea-pig eosinophils were resuspended (5×10^6 cells ml^{-1}) in PBS and aliquots (300 μl) of cells were dispensed into siliconized cuvettes which were placed into a dual channel platelet aggregometer (Chronolog 440 VS) linked to a dual pen recorder (Chronolog 707). The cells were incubated for 5 min at 37°C with continuous stirring at 700 r.p.m. before stimulation with the indicated agonist. The reference cuvette contained buffer alone. Responses were measured at the peak of aggregation and the results expressed as the percentage of maximal aggregation induced by 10^{-6} M phorbol myristate acetate (PMA). With the exception of salmeterol and dibutyryl cyclic AMP (dbcyclic AMP, 3 min pretreatment), eosinophils were pretreated with cyclic AMP-elevating agents for 2 min and then stimulated with PAF (10^{-8} M and 10^{-7} M) or C5a (10^{-7} M). The concentration of the agonists used was based on previous experiments which demonstrated that they elicited similar aggregation responses (Teixeira *et al.*, 1995a). For the experiments with the protein kinase A inhibitors, H89 and KT5720, eosinophils were pretreated with H89 (10^{-5} M) or KT5720 (10^{-6} or 3×10^{-6} M) for 3 min before the addition of PGE_1. Similarly, rolipram 10^{-7} M) was given 2 min before the addition of PGE_1 (10^{-10} to 10^{-8} M) or salbutamol (10^{-9} to 10^{-7} M).

Flow cytometric analysis of CD18 expression on eosinophils

Purified eosinophils (5×10^5 cells in 0.1 ml PBS/BSA 0.25%) were pre-incubated with control buffer or PGE_1 (10^{-7} M) for 2 min at 37°C and then activated with PAF (10^{-8} M), C5a (10^{-7} M) or PMA (10^{-7} M). After 2 min (PAF and C5a) or 10 min (PMA), a solution containing azide (0.1% final concentration) and an anti-CD18 mAb (6.5E, 50 μg ml^{-1} final concentration) was added and the cells left on ice for 15 min at 4°C. The cells were then washed twice with PBS, goat anti-mouse IgG antibody conjugated with flourescein isothiocyanate (FITC) was added (5 μl in 0.5 ml of cell suspension) and the cells were incubated for 15 min at 4°C. The preparations were washed twice and FITC fluorescence was determined on a Becton Dickinson FACScan flow cytometer (Oxford) and analysed by CELLQuest software. MOPC21 (mouse IgG_1) was used as a negative control.

Chemicals and antibodies

The following reagents were purchased from Sigma Chemical Company (Poole): bovine serum albumin (BSA), dibutyryl cyclic AMP (dbcyclic AMP), dimethyl sulphoxide (DMSO), D-glucose, 4β-phorbol myristate acetate (PMA), prostaglandin (PGE_1), PGE_2 and goat anti-mouse IgG FITC conjugate. Horse serum, Dulbecco's phosphate buffered saline (PBS, calcium- and magnesium-free, pH 7.4) and HBSS were from Life Technologies Ltd (Paisley). Percoll was from Pharmacia (Milton Keynes). C16 PAF was from Bachem (Saffron Walden) and KT5720 (8R*,95*,115*)-(−)-9-hydroxy-9-m-hexyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2, 7b,11a-triazadibenzo (a,g)cycloocta (cde)-frinden-1-one from Calbiochem (Nottingham). Recombinant human C5a was a gift from Dr J van Ossterum, Ciba Geigy (Summit, NJ, U.S.A.). The anti-CD18 mAb (6.5E, mouse IgG_1) and the control mAb MOPC21 (mouse IgG_1) were a gift from Dr M. Robinson, Celltech Ltd (Slough) and iloprost from Dr F. McDonald (Schering AG, Germany). Rolipram was a gift of Sandoz (Basel, Switzerland). Rolipram was dissolved in 100% ethanol (2.5 mg ml^{-1}) and further diluted in PBS. Salbutamol sulphate was purchased from Allen & Hanburys (Uxbridge). Salmeterol base (Ball *et al.*, 1991) was synthesized and kindly supplied by Ciba Geigy (Basel, Switzerland) as a racemic mixture. Salmeterol was dissolved in 100% DMSO (6 mg ml^{-1}) and diluted further in PBS. KT5720 was dissolved in ethanol and further diluted in PBS. H89 [N-[a-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide was purchased from Biomol (Nottingham) and was dissolved in 50% ethanol (5 mg ml^{-1}). None of the vehicles used in this study significantly altered eosinophil aggregation induced by C5a or PAF (data not shown).

Statistical analysis

Results were analysed by analysis of variance (ANOVA) followed by Student-Newman-Keuls post-test with the statistical progam Instat (GraphPad Software V2.03, CA, U.S.A.). When only two groups were compared, Student's *t* test was carried out. Results were considered significant when $P<0.05$. Data are presented as the means ± s.e.mean of *n* experiments.

Results

Initial experiments were carried out with the cyclic AMP permeant analogue dbcyclic AMP. When eosinophils were pretreated for 3 min with dbcyclic AMP (2.6×10^{-3} M), aggregation induced by PAF was inhibited by 43% (PAF 10^{-8} M, 30.8 ± 5.7% maximal aggregation; PAF + dbcyclic

AMP, 17.5±3.3%; $n=5$, $P<0.05$). These initial experiments suggested that elevating cyclic AMP may modulate eosinophil homotypic aggregation induced by some inflammatory mediators *in vitro*.

Effects of prostaglandins on eosinophil homotypic aggregation

As shown in Figure 1a, eosinophil homotypic aggregation induced by PAF (10^{-8} M) was completely inhibited by PGE_1 with an IC_{50} of approximately 2×10^{-9} M. Eosinophil aggregation in response to C5a (10^{-7} M) was also inhibited by PGE_1 but complete suppression of the response was not achieved (maximal inhibition was 67% at 10^{-6} M). In addition, PGE_1 was less potent at inhibiting C5a-induced eosinophil aggregation (IC_{50} $\sim 6 \times 10^{-8}$ M). In contrast, aggregation induced by PMA (10^{-8} to 10^{-6} M) was not inhibited by PGE_1 at any concentration examined (eg. PMA 10^{-6} M, 100% maximal aggregation: PMA + PGE_1 10^{-6} M, 107±5.7%, $n=4$). Typical traces showing the effects of increasing concentrations of PGE_1 on eosinophil aggregation induced by PAF are shown in Figure 1b.

PGE_2 (10^{-6} M) also inhibited PAF- and C5a-induced aggregation (Figure 2a) whereas the prostacyclin analogue iloprost (10^{-6} M) was inactive (Figure 2b).

Effects of β_2-adrenoceptor agonists on eosinophil homotypic aggregation

The concentration-dependent effects of salbutamol (10^{-8} to 10^{-5} M) on eosinophil aggregation induced by PAF and C5a are shown in Figure 3. Consistent with the PGE_1 results described above, salbutamol was more potent and more effective at inhibiting PAF- than C5a-induced responses (Figure 3). Thus, PAF-induced aggregation was inhibited by up to 76% with an IC_{50} of approximately 10^{-7} M and C5a-induced aggregation was inhibited by up to 52%.

The long-acting β_2-adrenoceptor agonist salmeterol (Ball *et al.*, 1991) also inhibited eosinophil aggregation induced by PAF and C5a when given as a 3 min pretreatment (Table 1) but inconsistently modified the responses when shorter pretreatment periods were used (data not shown). Since high concentrations of salmeterol have been previously shown to affect inflammatory cell function in a manner independent of β_2-adrenoceptor activation (Baker & Fuller, 1990), eosinophils were pretreated with propranolol for 2 min before the addition of salmeterol. As shown in Figure 4, propranolol (10^{-5} M) effectively reversed the inhibitory effects of salmeterol on PAF- (Figure 4a) and C5a-

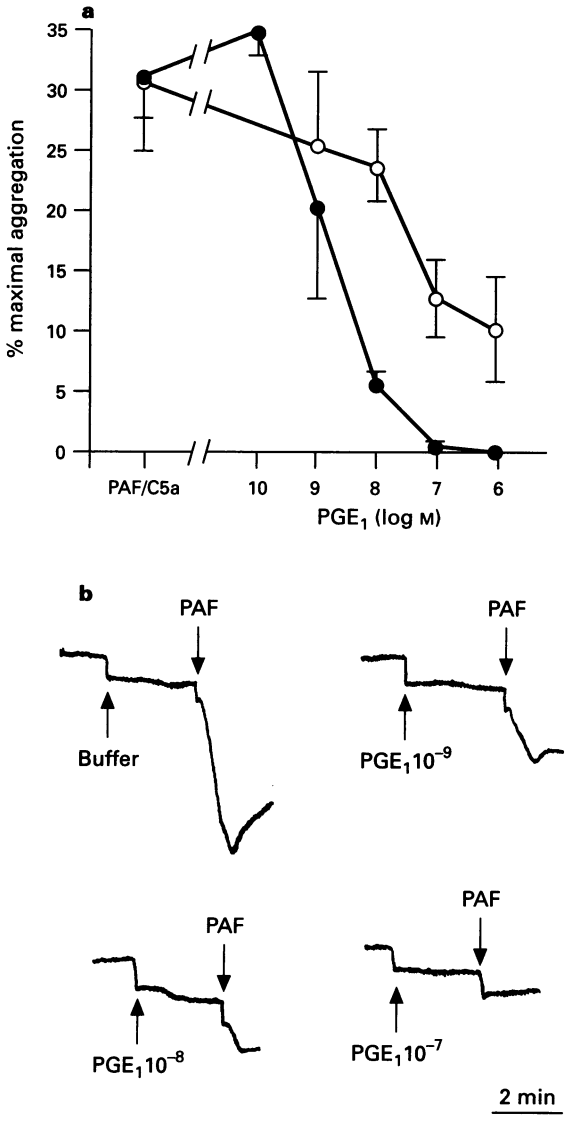

Figure 1 (a) Effect of prostaglandin E_1 (PGE_1) on eosinophil homotypic aggregation induced by PAF and the complement fragment C5a. Eosinophils were pretreated for 2 min with PGE_1 (10^{-10} M to 10^{-6} M) and then activated with PAF (10^{-8} M, ●) or C5a (10^{-7} M, ○). Results are expressed as the percentage maximal response induced by 4β-phorbol myristate acetate (10^{-6} M) and each point is the mean±s.e.mean (vertical lines) for 4 experiments. (b) Typical aggregation traces after activation of eosinophils with PAF (10^{-8} M) in control and PGE_1-treated cells (concentration shown as molar).

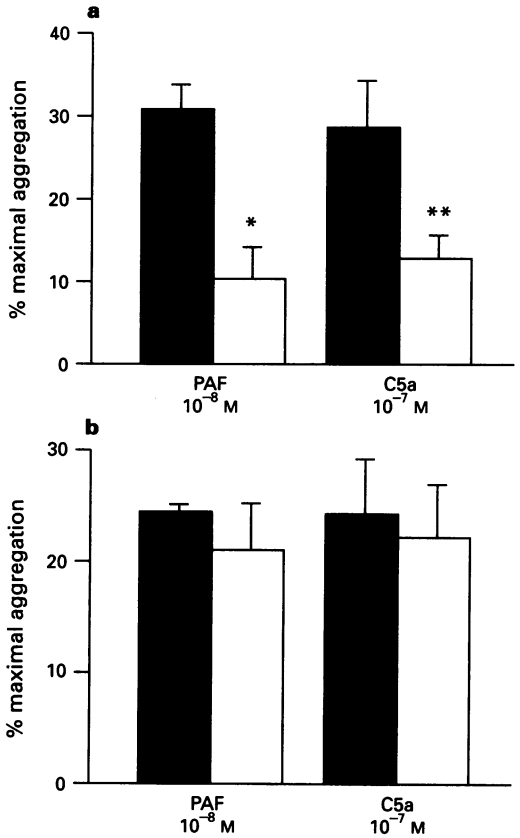

Figure 2 Effect of prostaglandin E_2 (PGE_2) (a) and iloprost (b) on eosinophil homotypic aggregation induced by PAF and the complement fragment C5a. Eosinophils were pretreated for 2 min with vehicle (solid columns), (a) PGE_2 (10^{-6} M, open columns) or (b) iloprost (10^{-6} M, open columns) and then activated with PAF or C5a. Results are expressed as the percentage maximal response induced by 4β-phorbol myristate acetate (10^{-6} M) and each point is the mean±s.e.mean (vertical lines) for 4 experiments. *$P<0.05$ and **$P<0.01$, respectively, when compared to control responses.

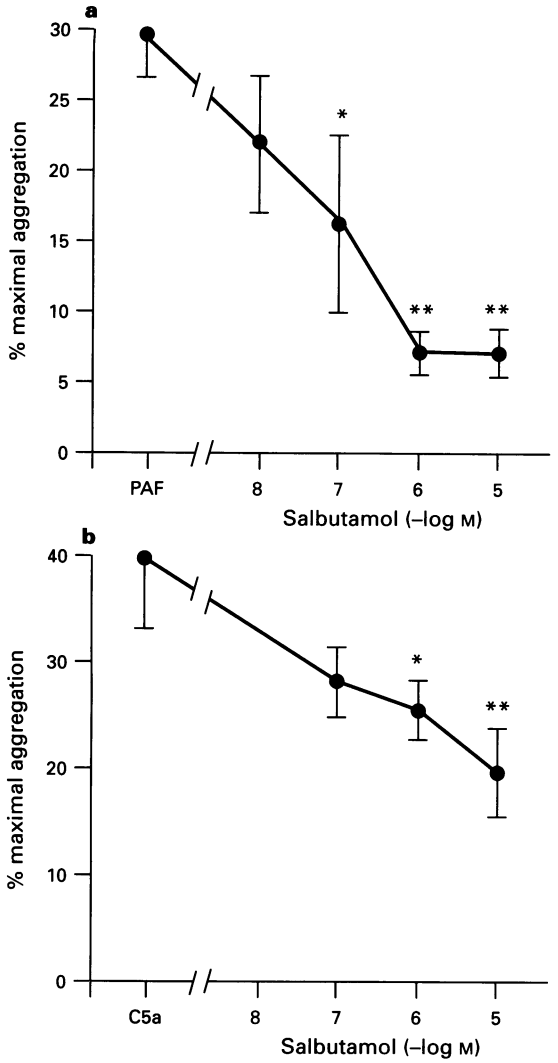

Figure 3 Effect of salbutamol on eosinophil homotypic aggregation induced by PAF (a) and the complement fragment C5a (b). Eosinophils were pretreated for 2 min with salbutamol (10^{-8} M to 10^{-5} M) and then activated with PAF (10^{-8} M, a) or C5a (10^{-7} M, b). Results are expressed as the percentage maximal response induced by 4β-phorbol myristate acetate (10^{-6} M) and each point is the mean ± s.e.mean (vertical lines) for 4 experiments. $^{*}P<0.05$ and $^{**}P<0.01$, respectively, when compared to control responses.

induced eosinophil aggregation (Figure 4b) suggesting that salmeterol was indeed acting via β-adrenoceptors.

Effects of a PDE4 inhibitor on eosinophil aggregation

Rolipram (10^{-7} to 10^{-5} M) inhibited PAF-induced eosinophil aggregation only at the highest concentration tested (Figure 5). At this concentration, PAF-induced responses were inhibited by 53% whereas C5a-induced responses were inhibited by 32% (Figure 5). Interestingly, pretreatment of eosinophils with a concentration of rolipram (10^{-7} M) that alone failed to affect PAF-induced eosinophil aggregation increased the potency of PGE_1 (10^{-10} to 10^{-8} M; Figure 6a) and salbutamol (10^{-9} to 10^{-7} M; Figure 6b) by approximately 10 fold.

Effects of protein kinase A inhibition

In order to assess the role of protein kinase A (PKA) in the inhibitory effects of cyclic AMP-elevating agents on eosinophil aggregation, we investigated the effects of the PKA inhibitors H89 and KT5720. H89 (10^{-5} M) had no significant effect on eosinophil aggregation induced by PAF (10^{-7} M; Figure 7a). However, when eosinophils were pretreated with the compound before the addition of PGE_1 (10^{-8} M), H89 completely reversed the inhibitory effects of PGE_1 on PAF-induced eosinophil homotypic aggregation (Figure 7). When a higher concentration of PGE_1 (10^{-7} M) which induced more

Table 1 Effect of salmeterol on eosinophil aggregation induced by PAF and C5a

	Inhibition of control responses (%) *Salmeterol (M)*				
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
PAF	71.6± 11.9**	79.2± 2.3**	70.2± 3.6**	45.2± 10.6**	29.9± 4.9*
C5a	33.1± 9.1**	48.8± 8.4**	13.7± 4.4	ND	ND

Eosinophils were pretreated with the indicated concentrations of salmeterol for 3 min and then activated with PAF (10^{-8} M) or C5a (10^{-7} M). Results are presented as the mean ± s.e.mean of 4 to 7 experiments. Eosinophil aggregation of cells treated with vehicle alone and then activated with PAF and C5a was 29.8 ± 2.5 and 27.6 ± 2.0% maximal aggregation, respectively. $^{*}P<0.05$ and $^{**}P<0.01$, respectively, when compared to control.

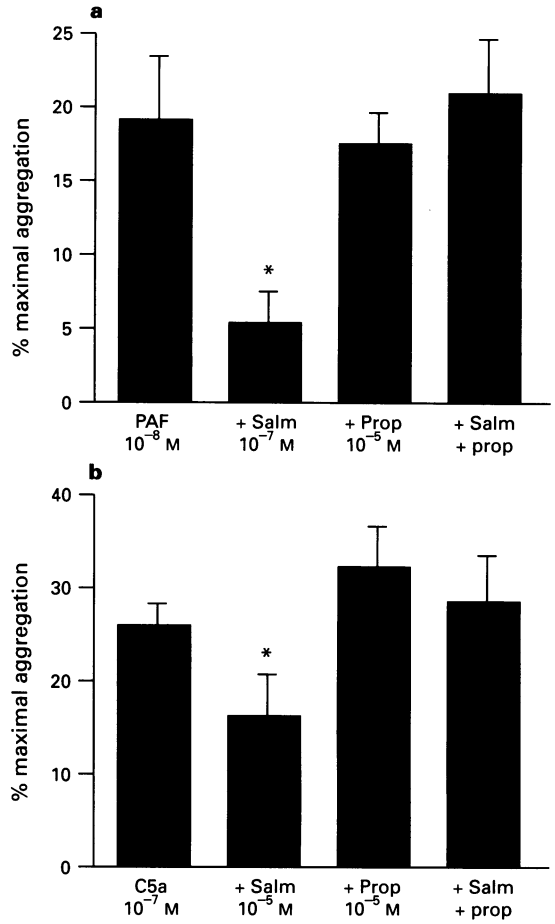

Figure 4 Reversal by propranolol (Prop) of the inhibitory effects of salmeterol (Salm) on PAF- and C5a-induced eosinophil homotypic aggregation. Eosinophils were pretreated for 3 min with salmeterol (10^{-7} M or 10^{-5} M) and then activated with PAF (10^{-8} M) or C5a (10^{-7} M). Propranolol (10^{-5} M) was given 2 min before salmeterol. Results are expressed as the percentage maximal response induced by 4β-phorbol myristate acetate (10^{-6} M) and are the mean ± s.e.mean (vertical lines) for 4 experiments. $^{*}P<0.05$ when compared to responses in the presence of PAF or C5a alone.

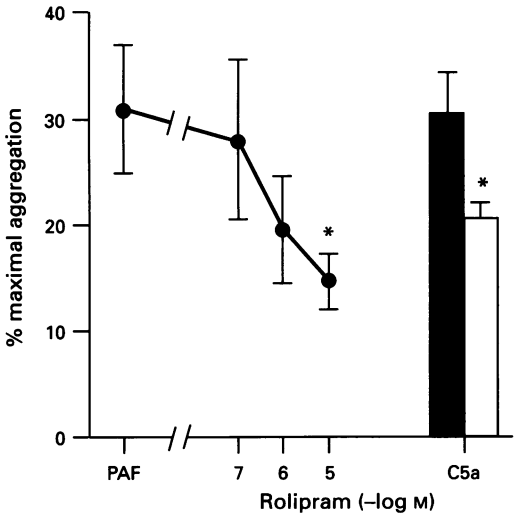

Figure 5 Effect of the PDE4 inhibitor rolipram on eosinophil aggregation induced by PAF and the complement fragment C5a. Eosinophils were pretreated for 2 min with rolipram (10^{-7} M to 10^{-5} M) and then activated with PAF (10^{-8} M). In addition, eosinophils were pretreated with vehicle (solid column) or rolipram (10^{-5} M, open column) and then activated with C5a (10^{-7} M). Results are expressed as the percentage maximal response induced by 4β-phorbol myristate acetate (10^{-6} M) and each point is the mean ± s.e.mean (vertical lines) for 4 experiments. *$P<0.05$ when compared to control responses.

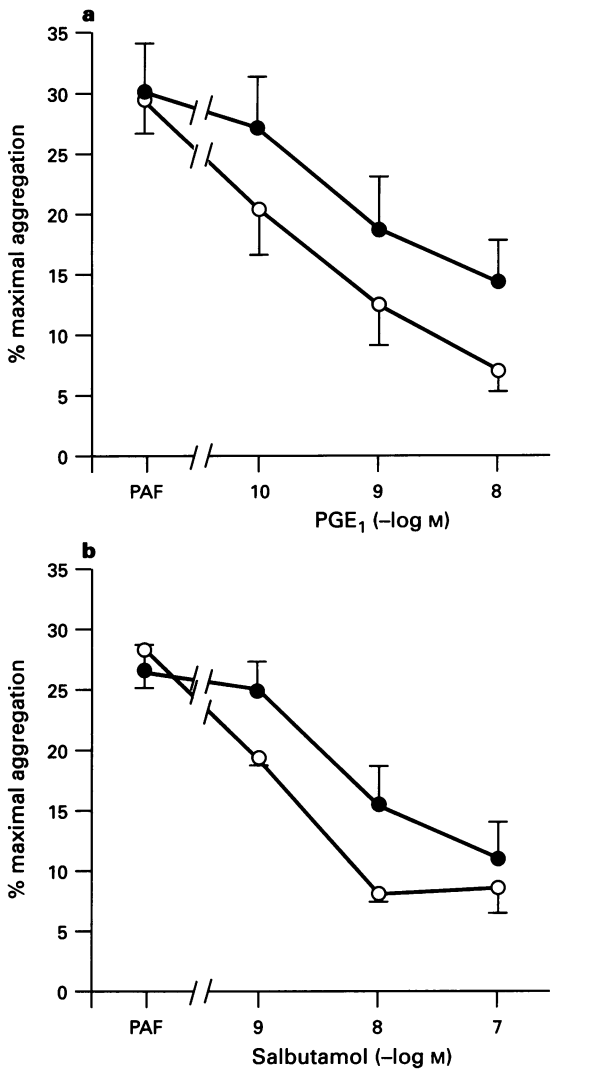

Figure 6 Effect of a low concentration of the PDE4 inhibitor rolipram on the inhibitory effects of (a) prostaglandin E_1 (PGE_1) and (b) salbutamol on eosinophil homotypic aggregation induced by PAF. Eosinophils were pretreated for 2 min with rolipram (10^{-7} M, ○) or control buffer (●). PGE_1 (10^{-10} M to 10^{-8} M) or salbutamol (10^{-9} M to 10^{-7} M) were then added for a further 2 min and the cells activated with PAF (10^{-7} M). Results are expressed as the percentage maximal response induced by 4β-phorbol myristate acetate (10^{-6} M) and each point is the mean ± s.e.mean (vertical lines) for 3–4 experiments. *$P<0.05$ when responses in the presence and absence of rolipram were compared.

complete inhibition of PAF-stimulated responses was used, H89 partially reversed the inhibitory effects of PGE_1, although this did not reach statistical significance (Figure 7a). KT5720 (3×10^{-6} M) produced effects qualitatively similar to H89 (Figure 7b. Thus, this compound significantly blocked the effects of 10^{-7} M PGE_1 and completely reversed the inhibitory effect of 10^{-8} M PGE_1 (Figure 7b). In contrast to H89, KT5720 significantly enhanced aggregation in response to PAF alone (Figure 7b).

Effects of PGE_1 on the expression of CD18 by eosinophils

In the present study, guinea-pig eosinophils had a high basal expression of CD18 (MOPC21, 6.4 ± 1.5 mean fluorescence intensity (MFI); 6.5E, 1157 ± 81 MFI, $n=4$) in agreement with our previous studies (Teixeira *et al.*, 1996). After activation with C5a (10^{-7} M) or PAF (10^{-8} M) there was a small but significant increase in MFI for 6.5E (Table 2). PGE_1 (10^{-7} M) inhibited by 62% and 93% this upregulation of CD18 induced by C5a and PAF, respectively (Table 2).

Discussion

When activated *in vitro* with different inflammatory stimuli, guinea-pig eosinophils undergo a concentration-dependent aggregation response (Teixeira *et al.*, 1995a; 1996). Eosinophil aggregation is dependent on calcium and magnesium ions and is largely dependent on CD18 present on the eosinophil surface (Teixeira *et al.*, 1995a; 1996). Human eosinophils also undergo homotypic aggregation *in vitro* when activated with inflammatory mediators (Koenderman *et al.*, 1991). The functional relevance of eosinophil aggregation *in vivo* is less clear but this phenomenon has been observed after i.d. injection of the cytokine RANTES in dog skin (Meurer *et al.*, 1993) and around migrating larvae of parasites (McLaren, 1980). In addition, because aggregation is dependent on CD18 present on the eosinophil surface (Teixeira *et al.*, 1995a), the study of eosinophil aggregation may shed light on the functional importance of this molecule and how it can be modulated pharmacologically. Indeed, we and others have demonstrated previously the importance of CD18 for eosinophil migration *in vivo* (Milne & Piper, 1994; Teixeira 1994a; Das *et al.*, 1995).

In this study we have assessed the effects of agents known to elevate cyclic AMP on the homotypic aggregation of eosinophils following activation with two inflammatory mediators, PAF and C5a. These mediators were chosen because of their ability to activate eosinophils *in vitro* and to induce their recruitment *in vivo* (Teixeira *et al.*, 1993; 1995a; Zech-Kapp *et al.*, 1995). Representatives from three classes of cyclic AMP elevating agents were used: prostaglandins, β_2-adrenoceptor agonists and a PDE4 inhibitor.

It is now widely acknowledged that the PDE4 isoenzyme family plays an important role in the regulation of eosinophil function *in vitro* and *in vivo* (Dent *et al.*, 1991; 1994; Giembycz, 1992; Hatzelmann *et al.*, 1995). For example, rolipram and related drugs effectively suppress activation of the NADPH oxidase (Dent *et al.*, 1991; 1994; Barnette *et al.*, 1995), prostanoid generation (Souness *et al.*, 1994) and degranulation

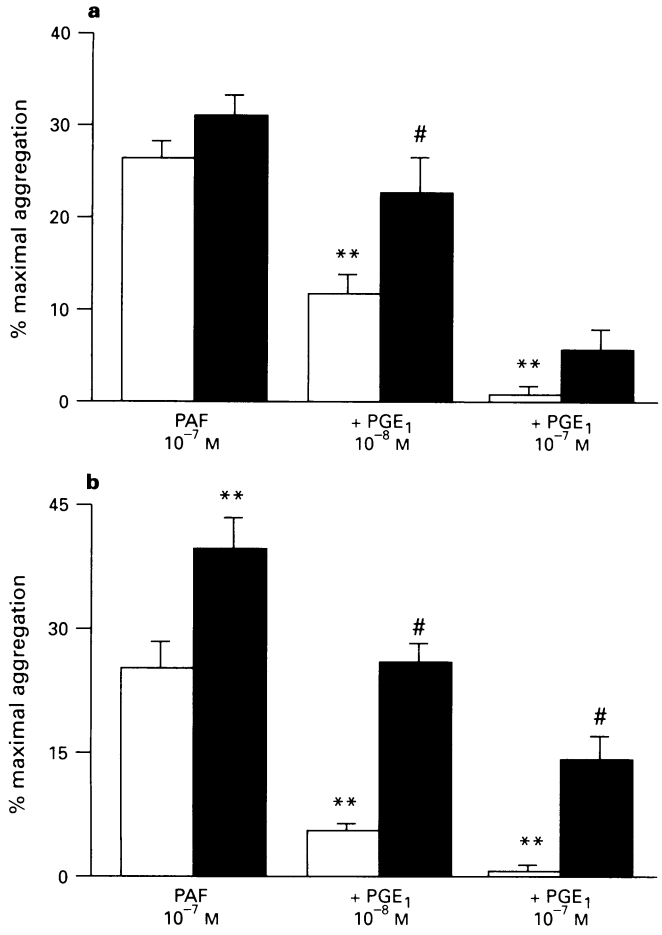

Figure 7 Reversal by the protein kinase A inhibitors (a) H89 and (b) KT5720 of the inhibitory effects of prostaglandin E_1 (PGE_1) on PAF-induced eosinophil homotypic aggregation. Eosinophils were pretreated for 2 min with PGE_1 (10^{-8} M), PGE_1 (10^{-7} M) or vehicle (shown as PAF) and then activated with PAF (10^{-7} M). The protein kinase A inhibitors (a) H89 (10^{-5} M, solid columns) or (b) KT5720 (3×10^{-6} M, solid columns) were given 3 min before PGE_1 or vehicle. Results are expressed as the percentage maximal response induced by 4β-phorbol myristate acetate (10^{-6} M) and each point is the mean ± s.e.mean (vertical lines) for 4 experiments. $^*P<0.01$ when compared to responses induced by PAF alone. $^{\#}P<0.05$ when compared to responses in the presence of PAF and PGE_1.

Table 2 Effect of PGE_1 on the magnitude of CD18 expression by eosinophils activated with PAF and C5a

	Mean fluorescence intensity	
	Buffer	*PGE_1*
Unstimulated	1157 ± 81	1202 ± 64
PAF	1378 ± 126#	1217 ± 107*
C5a	1452 ± 28#	1315 ± 89*

Eosinophils were incubated with buffer or PGE_1 (10^{-7} M) for 2 min and then activated for 2 min with PAF (10^{-8} M) or C5a (10^{-7} M). Results are presented as the mean ± s.e.mean of 4 experiments. $^*P<0.05$ when compared to unstimulated cells and $^{\#}P<0.05$ when compared to cells treated with PAF or C5a alone.

(Hatzelmann *et al.*, 1995). Similarly, in various animal models, PDE4 inhibitors attenuate pulmonary and cutaneous eosinophil recruitment in response to a wide range of stimuli (Howell *et al.*, 1993; Underwood *et al.*, 1993; 1994; Teixeira *et al.*, 1994b). The results presented herein are consistent with these data and extend the spectrum of activity of rolipram to include the inhibition of eosinophil homotypic aggregation.

PGE_1 effectively inhibited PAF- and C5a-induced aggregation. Although not formally addressed in this study, it is likely that this effect is mediated via EP_2- or EP_4-receptors which couple positively to adenylyl cyclase. Evidence to support this contention is two fold: first, PGE_2 increases cyclic AMP in guinea-pig eosinophils, an affect that is potentiated by PDE4 inhibitors (Souness & Scott, 1993), and second EP_2-receptors predominate on human eosinophils (Butcher & Vardey, 1990). The lack of effect of iloprost on eosinophil aggregation suggests that EP_1- and IP-receptors are not expressed by guinea-pig eosinophils or that their activation has no role at inhibiting aggregation. However, further studies with selective agonists and antagonists are clearly necessary to identify and classify the prostanoid receptors expressed by these cells.

Interestingly, pretreatment of eosinophils with the PKA inhibitors H89 and KT5720 completely reversed the inhibitory effects of a low (10^{-8} M) but not a maximally effective (10^{-7} M) concentration of PGE_1 on PAF-induced aggregation. While these finding may reflect incomplete inhibition of PKA by H-89 and KT-5720, these data, nevertheless, suggest that activation of the cyclic AMP/PKA cascade plays an important role in preventing the aggregation response. This conclusion is supported further by the finding that a concentration of the PDE4 inhibitor, rolipram, which did not inhibit aggregation *per se*, produced a parallel, leftwards shift in the PGE_1 concentration-response curve (see Figure 6). These data are entirely consistent with a fundamental pharmacological principle which proposes that inhibitors of cyclic nucleotide PDEs should interact in a synergistic manner with activators of adenylyl (and guanylyl) cyclase.

Two β_2-adrenoceptor agonists, salbutamol and salmeterol, were evaluated for their inhibitory effects on eosinophil homotypic aggregation. The two compounds inhibited both PAF- and C5a-induced eosinophil aggregation in a concentration-dependent manner, but eosinophils needed to be pretreated with salmeterol for longer (3 min) for inhibition to be observed. This observation is consistent with the delayed onset of action of the compound in other tissues (Ball *et al.*, 1991). While salbutamol has been previously demonstrated to suppress various indices of eosinophil activation including lipid mediator release and free radical generation (Rabe *et al.*, 1993; Munoz *et al.*, 1994; Dent *et al.*, 1995), the ability of salmeterol similarly to inhibit eosinophil aggregation was slightly unexpected. Indeed, in previous studies salmeterol displays little, if any, agonist activity on eosinophils (Rabe *et al.*, 1993; Munoz *et al.*, 1995) and, in fact, behaves as a competitive antagonist at β-adrenoceptors with a pA_2 of ~6 (Rabe *et al.*, 1993). While the explanation for this discrepancy is unclear, it is not due to a β-adrenoceptor-independent action as the inhibition of aggregation effected by salmeterol was effectively antagonised by propranolol. It is possible that the nature and concentration of the stimulus may determine whether salmeterol exhibits agonist activity. Equally, eosinophil aggregation may be more sensitive to inhibition by cyclic AMP compared with degranulation and free radical generation. Clearly, further studies are required to resolve this intriguing anomaly.

The ability of salbutamol to suppress eosinophil aggregation was significantly potentiated in the presence of a threshold concentration of rolipram. These data were qualitatively identical to the results obtained with PGE_1 under identical experimental conditions and corroborate the findings of Hatzelmann *et al.* (1995) where rolipram potentiates the inhibitory effect of salbutamol on eosinophil degranulation. Collectively, therefore, these data provide persuasive pharmacological evidence that salbutamol also inhibits eosinophil aggregation by a cyclic AMP-dependent mechanism.

We have previously shown an anti-CD18 monoclonal antibody to inhibit eosinophil aggregation induced by various stimuli by up to 70% (Teixeira *et al.*, 1995a; 1996). In order to investigate whether modulation of the number of CD18 sites on the eosinophil surface played any role on the inhibitory

effects of cyclic AMP elevating agents on aggregation, eosinophils were evaluated for CD18 expression by flow cytometric analysis. Although there are data demonstrating a role for elevated levels of cyclic AMP in the control of the expression of CD18 by neutrophil (Derian *et al.*, 1995), there are few data regarding eosinophils. Guinea-pig peritoneal eosinophils express high numbers of CD18-binding sites on their surface (see Table 2), although when activated with PAF and C5a these were significantly upregulated. Because PGE_1 was an effective inhibitor of eosinophil aggregation, this cyclic AMP-elevating agent was used in this part of the study. Interestingly, when the cells were pretreated with PGE_1, the magnitude of CD18 increase was reduced after activation with both C5a or PAF. It is not clear whether such small alterations in the number of CD18 on the eosinophil surface would be sufficient to account for eosinophil aggregation observed after activation with PAF or C5a; an increase in the affinity of CD18 already present on the eosinophil surface may account for the functional responses observed (Diamond & Springer, 1994). Due to the unavailability of appropriate reagents, we were not able to measure changes in affinity of guinea-pig eosinophil CD18, although it has been previously shown that cyclic AMP-elevating agents are capable of inhibiting, at least in human lymphocytes, the increase in affinity of CD11a after stimulation (Dustin & Springer, 1989). These results suggest that cyclic AMP-elevating agents, such as PGE_1, inhibit eosinophil aggregation at least partially by inhibiting the upregulation of CD18 molecules on the eosinophil surface or, possibly, by inhibiting the increase in affinity of CD18 after activation.

An interesting observation was the consistent ability of cyclic AMP-elevating agents to inhibit PAF-induced aggregation with greater potency and efficacy than responses induced by C5a. These results suggest that these two mediators induce aggregation by interacting with different signal transduction pathways which are differentially modulated by cyclic AMP-elevating agents. We are at present investigating this possibility.

We have previously shown that cyclic AMP-elevating agents such as prostaglandins (Teixeira *et al.*, 1993), β_2-adrenoceptor agonists (Teixeira *et al.*, 1994b) and PDE4 inhibitors (Teixeira *et al.*, 1995b) effectively suppress eosinophil recruitment *in vivo*. In our *in vivo* model, PGE_1 and PGE_2, but not iloprost, effectively inhibit eosinophil accumulation (Teixeira *et al.*, 1993). These *in vivo* findings are remarkably similar to our present findings with eosinophil aggregation *in vitro*. Together these studies suggest that drugs which elevate cyclic AMP are effective at suppressing eosinophil recruitment to sites of inflammation *in vivo* and that the eosinophil itself, through decreased expression of CD18 or decreased affinity for CD18, may be an important cellular target for the action of these drugs. In addition, cyclic AMP-elevating agents also inhibit other eosinophil functional responses (eg. respiratory burst, lipid production) which gives further support for the use of these agents in the therapy of allergic diseases such as asthma.

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