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Requirement of additional adenylate cyclase activation for the inhibition of human eosinophil degranulation by phosphodiesterase IV inhibitors

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Abstract

Human eosinophils contain predominantly phosphodiesterase type IV, but selective inhibitors of this isoenzyme fail to inhibit certain eosinophil responses such as degranulation. In this study, the effect of activation of adenylate cyclase on the ability of several highly selective PDE IV inhibitors to inhibit complement C5a-induced O_2^- release and degranulation of human eosinophils in vitro was investigated. All four selective PDE IV inhibitors, *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxybenzamide (RP 73401), rolipram, *N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indol-3-yl]glyoxylacidamide (AWD 12-281) and c-4-cyano-4-(3-cyclopentyloxy-4-methoxyphenyl-r-1-cyclohexane carboxylic acid) (SB 207499) potently inhibited C5a-induced O_2^- generation (IC_{50} = 0.03, 0.42, 0.55 and 0.86 μM, respectively), but generally failed to inhibit degranulation. The only exception was AWD 12-281, which inhibited degranulation (IC_{50} = 16.2 μM). In the presence of different AC activators (histamine, salbutamol, prostaglandin E_2 and forskolin), the PDE IV inhibitors became potent inhibitors of degranulation. The interaction between the PDE IV inhibitors and the AC activators resulted in a synergistic increase in intracellular levels of adenosine 3′, 5′-monophosphate (cAMP). These results show that PDE IV inhibitors generally require an additional cAMP signal to be able to inhibit eosinophil degranulation, and that this signal can be generated via both membrane receptors and direct AC activation. This may be relevant to the in vivo effectiveness of PDE IV inhibitors in eosinophilic inflammation. © 2001 Published by Elsevier Science B.V.

Keywords: Eosinophil; Degranulation; Phosphodiesterese IV inhibitors; cAMP; Adenylate cyclase; Synergism

1. Introduction

Adenosine 3′,5′-cyclic monophosphate (cAMP) is an important intracellular messenger that regulates many cellular activities. In many pro-inflammatory and immune cells, an increase in the intracellular concentration of this nucleotide generally leads to inhibition of responses (Dent et al., 1991; Fonteh et al., 1993; Prabhakar et al., 1994). For this reason, agents that elevate intracellular cAMP have potential as anti-inflammatory and anti-allergic drugs. Elevation of intracellular cAMP levels can be achieved through the activation of adenylate cyclase (adenylyl cyclase; EC 4.6.1.1) either directly or through appropriately coupled membrane receptors, as well as by preventing the hydrolysis of cAMP by the cyclic nucleotide phosphodiesterases (3′, 5′ cyclic nuclotide 5′-nucleotidohydrolase; EC 3.1.4.17).

PDEs represent a large number of isoenzymes which have been grouped into seven isoenzyme families (PDE 1 to PDE VII) based on the encoding gene or gene family as well as other criteria such as substrate specificity and kinetics, tissue distribution and sensitivity to inhibitors (Beavo, 1995; Conti et al., 1995; Loughney and Ferguson, 1996). Although many cell types may contain more than one PDE isoenzyme, PDE IV appears to be the predominant isoform in virtually all inflammatory cells (Torphy and Undem, 1991; Giembycz, 1992). Indeed, PDE IV inhibitors have been reported to be effective down-regulators of the various responses of pro-inflammatory cells, including eosinophils (Semmler et al., 1993; Torphy et al., 1994; Hatzelmann et al., 1995; Au et al., 1998).

In the pathophysiology of allergic disease, especially asthma, eosinophils are known to play important roles (Frigas and Gleich, 1986; Barnes, 1989). They infiltrate the asthmatic lung where they release tissue-damaging granular proteins such as the major basic protein and eosinophil peroxidase, lipid mediators such as the leuko-

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trienes, as well as oxygen-free radicals which, in concert, orchestrate bronchial inflammation and subsequent bronchial hyperresponsiveness (Laitinen et al., 1985; Motijima et al., 1989).

The promise of PDE IV inhibitors as effective anti-asthma drugs is based mainly on their anti-inflammatory effect (Schudt et al., 1995; Giembycz, 2000), and a major component of this effect is the ability to inhibit eosinophil responses. In guinea pigs, PDE IV inhibitors have been reported to inhibit the respiratory burst and degranulation of eosinophils (Dent et al., 1991; Souness et al., 1991; 1995) as well as their infiltration into allergen-challenged lungs (Underwood et al., 1993; Banner and Page 1995). Only few studies have addressed the effect of PDE inhibitors on human eosinophil function. While both non-selective PDE inhibitors such as theophylline and selective PDE IV inhibitors such as rolipram have been reported to inhibit the human eosinophil respiratory burst and chemotaxis (Dent et al., 1994; Cohan et al., 1996), only non-selective PDE inhibitors appear to be able to inhibit eosinophil degranulation (Hatzelmann et al., 1995; Ezeamuzie and Al-Hage, 1998). In their studies, Hatzelmann et al. (1995) have shown that in the presence of the β_2-adrenoceptor agonist, salbutamol, the selective PDE IV inhibitors became effective inhibitors of eosinophil degranulation. It was not determined, however, whether such potentiation was a consequence of a parallel elevation of intracellular cAMP or whether other methods of activating adenylate cyclase were equally effective in potentiating the inhibitory effect. The purpose of the present work was, therefore, to investigate the requirement for an additional cAMP signal by a number of PDE IV inhibitors *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxybenzamide (RP 73401), rolipram, *N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indol-3-yl]glyoxylacidamide (AWD 12-281) and c-4-cyano-4-(3-cyclopentyloxy-4-methoxyphenyl-r-1-cyclohexane carboxylic acid) (SB 207499) for the inhibition of complement C5a-induced degranulation of human eosinophils in vitro. It also aimed at determining whether such a signal can be generated by other means of activating adenylate cyclase.

2. Materials and methods

2.1. Isolation of blood eosinophils

Fresh blood was obtained from consenting healthy adults or mildly atopic individuals who have no allergic disease. Eosinophils were purified by a slight modification of the immunomagnetic method (Hansel et al., 1991). Briefly, three parts of sodium citrate–anti-coagulated (13 mM final) blood was mixed with one part of 1% (w v^{-1} of 0.9% saline) hydrated methylcellulose solution to sediment the erythrocytes over 30 min at room temperature. The leucocyte-rich supernatant was collected and the leucocytes were recovered by centrifugation. After aspiration of the platelet-rich supernatant, the pelleted leucocytes were washed in "wash buffer" (Ca^{2+}- and Mg^{2+}-free, 10 mM HEPES-buffered Hanks balanced salt solution containing 0.25% bovine serum albumin) and resuspended in the same buffer at approximately 10% of the original blood volume. A 2-ml aliquot was then layered on a two-step Percoll gradient (1.080 and 1.090 g ml^{-1}) and centrifuged at $900 \times g$ on a Beckman (GS-6R) centrifuge for 20 min at room temperature. The upper layers (mononuclear cells and Percoll) were discarded and the pellet (granulocytes) was recovered and washed twice in the same buffer by centrifugation at $600 \times g$ for 10 min at 4°C. After hypotonic lysis of contaminating erythrocytes with ice-cold distilled water and readjustment of the tonicity with the same volume of double-strength saline, the cells were washed, counted and resuspended at a concentration of 2×10^7 cells ml^{-1} in the wash buffer. For eosinophil purification, 1.25 ml of the granulocyte suspension was then mixed with 5-μl mouse anti-human CD16 monoclonal antibody in a siliconized test tube, and incubated on ice for 1 h with frequent gentle rotation. Cells were then washed twice in wash buffer and then resuspended in 500 μl of prewashed immunomagnetic beads, pre-coated with affinity-purified sheep anti-mouse immunoglobulin G (2×10^8 coated beads), and incubated in ice for 1 h with frequent tube rotation. The immunomagnetically labeled neutrophils were removed by magnetic extraction. The purified eosinophils were then recovered by centrifugation and resuspended in reaction buffer ("wash buffer" containing 2 mM Ca^{2+} and 1 mM Mg^{2+}) for experiments. The eosinophil purity was assessed by differential counting of a Wright–Giemsa-stained cytosmear. The final cell preparation routinely consisted of over 98% pure eosinophils. Viability was determined by Trypan blue exclusion and always exceeded 98%.

2.2. Superoxide anions release

Superoxide anion (O_2^-) generation was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sedgwick et al., 1988). Purified eosinophils were resuspended at a concentration of 5×10^5 cells ml^{-1}, and 50-μl aliquots were dispensed into each well of a 96-well microplate containing 50 μl of cytochrome *c* (100 μM, final) and 50-μl reaction buffer. After pre-warming of the mixture, 50 μl of the stimulus was then added and the mixture was incubated at 37°C for 1 h. Corresponding wells containing 0.6 μM (final) SOD were included to assess specific O_2^- formation. In experiments in which the effect of drugs was assessed, 50 μl of the drugs was added in place of the buffer and incubated with the cells for 10 min before the addition of the stimulus. After incubation, 150 μl was transferred to a fresh plate and the absorbance was read at 550 nm on a Titertek Multiscan (Flow Labs, Rickmansworth, UK) plate reader. The amount of O_2^- generated was estimated as nmol ferricytochrome *c* re-

duced/10^6 cells h^{-1}, using an extinction coefficient of 2.1×10^{-4} M^{-1} cm^{-1}.

2.3. Eosinophil peroxidase release

Fifty microlitres of pre-warmed cell suspension containing 2.5×10^4 cells was dispensed into each well of a microplate. Then, 100 μl of the reaction buffer containing 10 μg ml^{-1} cytochalasin B was added and, after a 10-min pre-incubation, the cells were stimulated with 50 μl of human recombinant C5a. The mixture was further incubated for 30 min at 37°C. It had been determined in pilot experiments that this time was sufficient for the virtual completion of the degranulation process. At the end of the incubation period, the reaction was stopped by cooling on ice and after centrifugation at $600 \times g$ for 10 min, 50-μl aliquots of the supernatant as well as Triton X-100-lysed cells (for total content determination) were taken for the determination of the released enzymes. EPO activity was measured by the O-phenylenediamine method as previously reported (Kroegel et al., 1989). Briefly, OPD substrate solution, containing 0.4 mg ml^{-1} OPD and 0.4 mg ml^{-1} urea hydrogen peroxide in phosphate buffered saline-citrate buffer (pH 4.5), was prepared from SIGMA FAST® OPD tablets. One hundred microlitres of this substrate was added to 50 μl of the samples in a microplate and incubated for 30 min at 37°C. After incubation, the reaction was then stopped with 50 μl of 4 M H_2SO_4 and the absorbance was read at 490 nm. Values are expressed as percentages of the total content, using the amount obtained in half the same number of cells, after lysis, as 50%. The recovery of released EPO activity was usually above 80% at the end of a 30-min incubation.

2.4. Intracellular cyclic AMP determination

One million purified eosinophils, resuspended in 100 μl of BSA-free reaction buffer, were dispensed into each well of a 96-well plate containing 50 μl of the test PDE inhibitor or vehicle and incubated for 10 min at 37°C. The reaction was then started by the addition of 50 μl of warmed stimuli (AC activators). Three minutes later—a time previously determined to be best for this response (10 min for forskolin)—the reaction was stopped by the direct addition of 22.2 μl of 1N HCl. After thorough mixing of the reaction mixture and further incubation for 10 min, the plate was centrifuged at $1500 \times g$ for 10 min and 200 μl of the supernatant was taken and stored at −43°C, pending cAMP assay.

Cyclic AMP levels were measured, after acetylation, using a commercially available enzyme-linked immunosorbent assay kit, and following the manufacturer's instructions. The sensitivity of the assay was 0.01 pmol/well.

2.5. Drugs and chemical reagents

The following drugs and reagents were obtained from Sigma, St Louis, MO, USA: recombinant human C5a, Percoll, HEPES buffer, BSA, OPD, dimethylsulphoxide (DMSO), 3-isobutyl-1-methylxanthine (IBMX), theophylline, histamine dihydrochloride, prostaglandin E_2, cytochalasin B and all inorganic salts. Other drugs were obtained as follows: AWD 12-281 (Arzneimittelwerk Dresden, Radebuel, Germany); RP 73401 (Rhone-Poulenc Rorer, Dagenham, UK); SB 207499 (SmithKline Beecham, King of Prussia, PA, USA); rolipram and salbutamol hemisulphate (Research Biochemicals, Natick, MA, USA) and mouse monoclonal anti-human CD16 antibody (clone FcR gran1) (CLB, Amsterdam, Netherlands. The magnetic beads (coated with sheep anti-mouse immunoglobulin G) were supplied by Dynal, Oslo, Norway, while the cAMP assay kit (direct method) was obtained from Assay Designs, Ann Arbor, MI, USA.

Stock solutions of PDE IV inhibitors were made in DMSO to concentrations in the range ($1–4 \times 10^{-1}$ M) and then diluted directly in buffer. Prostaglandin E_2 and forskolin were initially dissolved in ethanol while salbutamol was dissolved in 0.1 N HCl. The final concentration of the solvents at the highest drug concentrations did not exceed 0.05%—a concentration that has no effect on eosinophil responses. All the other drugs and reagents were first dissolved in distilled water and diluted in reaction buffer.

2.6. Statistical analysis

Experimental data are presented as means ± S.D. from the number (n) of independent experiments. The concentrations producing 50% inhibition (IC_{50} values) were calculated from the concentration–effect curves by non-linear regression analysis using Grap*h*Pad InPlot (GraphPad Software, Philadelphia, USA). Statistical significance (P) was determined by the unpaired *t*-test or by one-sample *t*-test as appropriate (InStat, Grap*h*Pad, Software, USA).

3. Results

3.1. Effect of selective and non-selective PDE inhibitors on O_2^- release and degranulation

In the absence of CB, C5a induced a pronounced and concentration-dependent release of O_2^- from purified eosinophils (data not shown). At the sub-optimal concentration of 10 nM, O_2^- release was equivalent to that reducing 45.8 ± 6.7 nmol cytochrome $c/10^6$ cells h^{-1}.

Pre-incubation of eosinophils with the 4 PDE IV inhibitors resulted in a potent and concentration-dependent inhibition of O_2^- release by all the agents (Fig. 1A). The

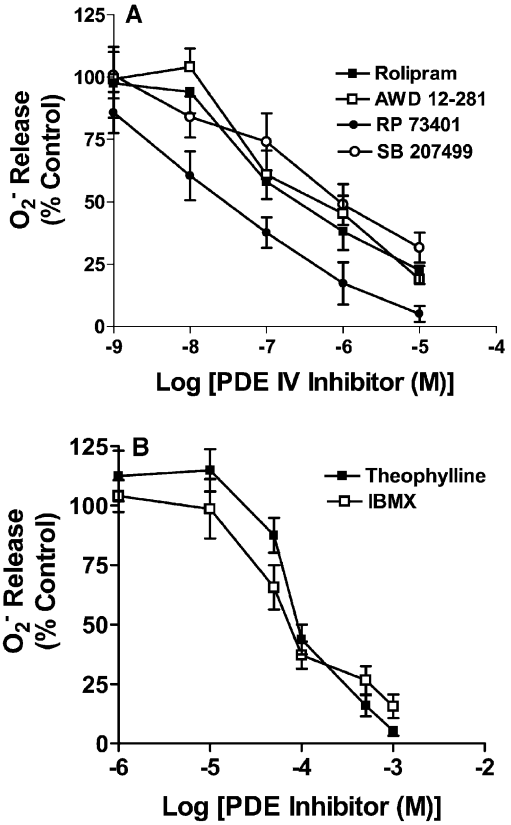

Fig. 1. The effect of the selective PDE IV inhibitors (A) and the non-selective PDE inhibitors (B) on O_2^- release from human eosinophils induced by C5a (10 nM). The uninhibited (control) release was equivalent to the amount reducing 45.8 ± 6.7 nmol cytochrome $c/10^6$ cells h^{-1}. Cells were incubated with the drugs for 10 min before stimulation. Values are means $\pm$ S.D., $n = 5$.

IC_{50} values (95% CI) were 0.03 (0.01–0.06) μM, 0.42 (0.22–0.70) μM, 0.55 (0.28–0.85) μM and 0.86 (0.48–1.36) μM, for RP 7341, rolipram, AWD 12-281 and SB 207499, respectively, $n = 5$. The non-selective PDE inhibitors theophylline and 3-isobutyl-1-methylxanthine (IBMX) were also effective, but the potency was considerably less (Fig. 1B)—the IC_{50} values (95% CI) being 78.4 (40.0–106.3) μM and 93.7 (62.2–126.8) μM, respectively, $n = 5$. At the concentrations tested, none of the PDE inhibitors had any significant oxygen radical-scavenging effect, as determined with the hypoxanthine–xanthine oxidase system.

In the presence of CB (5 μg ml^{-1}), the sub-optimal concentration of C5a (10 nM) induced the release of 13–45% of the total cell EPO content. In contrast to the effect of the PDE IV inhibitors on O_2^- release, pre-treatment of cells with these drugs was generally ineffective in inhibiting EPO release (Fig. 2A). The only exception was AWD 12-281, which produced a concentration-dependent inhibition that was statistically significant at concentrations above 1 μM. Its IC_{50} value (95% confidence interval) was 16.3 (9.3–22.8) μM, $n = 7$, and at 50 μM, the inhibition was ≈ 65%.

Unlike most of the selective PDE IV inhibitors, the non-selective PDE inhibitors, theophylline and IBMX, effectively inhibited EPO release (Fig. 2B). Their IC_{50} values (95% CI) were 246 (158.4–335.3) μM and 115.8 (78.3–142.6) μM, respectively, $n = 4$. At 1 mM, both drugs achieved almost complete abolition of degranulation. None of the inhibitors induced direct EPO release or affected EPO measurements.

3.2. The effect of activators of adenylate cyclase on the inhibition of EPO release by PDE inhibitors

To study the effect of the AC activators on the ability of PDE inhibitors to inhibit EPO release, the effects of these agents alone were first determined. As shown in Fig. 3, concentrations of salbutamol and forskolin up to 10 μM had no effect on EPO release. However, histamine and, to a lesser extent, prostaglandin E_2 were effective in inhibiting EPO release. The IC_{50} value (95% CI) for histamine was 1.5 (0.8–2.8) μM, while the inhibition by prostaglandin E_2, though statistically significant at 1–10 μM,

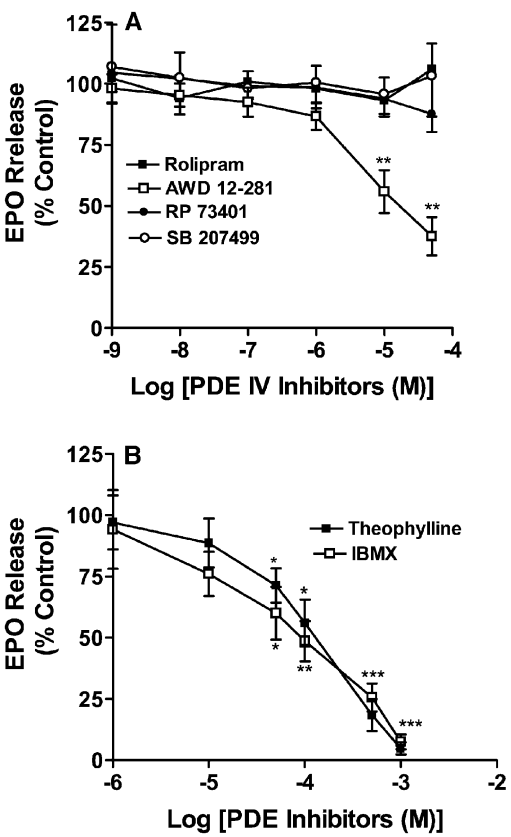

Fig. 2. The effect of the selective PDE IV inhibitors (A) and the non-selective inhibitors (B) on EPO release from human eosinophils induced by C5a (10 nM) in the presence of 5 μg ml^{-1} cytochalasin B. The uninhibited release was in the range 13–45% of the total cell content. Cells were incubated with the drugs for 10 min before stimulation. Values are means $\pm$ S.D., $n = 7$ for a, and $n = 4$ for b. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

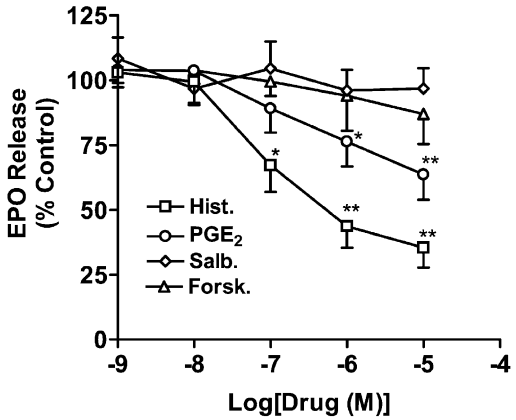

Fig. 3. The effect of adenylate cyclase activators (histamine, Prostaglandin E_2, salbutamol and forskolin) on EPO release from human eosinophils induced by C5a (10 nM) in the presence of 5 μg ml^{-1} cytochalasin B. The uninhibited release was in the range 13–45% of the total cell content. Cells were incubated with the drugs for 10 min before stimulation. Values are means ± S.D., $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$.

did not reach 50%. Using concentrations of these activators that had little or no effect of their own, the interaction with the PDE inhibitors on the inhibition of EPO release was subsequently studied. Rolipram and AWD 12-281 were used as prototypes of inactive and active PDE IV inhibitors, respectively, while theophylline was used as a prototype of a non-selective PDE inhibitor. As shown in Fig. 4A, in the presence of histamine (0.03 μM), prostaglandin E_2 (0.1 μM), salbutamol (1 μM) and forskolin (10 μM), the completely inactive rolipram became a highly potent inhibitor. Histamine and prostaglandin E_2 were the most effective in potentiating the rolipram effect, followed by salbutamol and lastly, forskolin. For example, the percentage inhibitions produced by histamine, prostaglandin E_2, salbutamol and forskolin alone at the above given concentrations were 17.3%, 11.4%, 9.7% and 8.7%, respectively, while the inhibition produced by rolipram (10 μM) alone was 1.8%. Together, the percentage inhibition (expected sum of the individual effects) was 64.0 (19.1)%, 58.8 (13.2)%, 54.2 (11.5)% and 42.6 (10.5)%, respectively. The combined effects were all significantly greater than the expected sum of the individual effects, $P < 0.05$–0.001, $n = 7$, thus indicating strong synergism.

Similar effects were seen with AWD 12-281 (Fig. 4B), but in this case prostaglandin E_2 was the most effective potentiator, followed by histamine. Again, the potentiating effect of forskolin was the weakest of the four AC activators—its interaction being essentially additive in this case. Interestingly, the synergistic interaction of AWD 12-281 with histamine, prostaglandin E_2 and salbutamol occurred even at concentrations of AWD 12-281 that produced a significant inhibition on their own. For theophylline, the interaction with all the AC activators was essentially additive at lower concentrations, but less than additive at higher concentrations (Fig. 4C).

3.3. Interaction between PDE inhibitors and AC activators on intracellular cAMP responses

Table 1 shows the results of the interaction between the PDE inhibitors and the AC activators on the intracellular cAMP response. In the absence of PDE inhibitors, the intracellular cAMP level in unstimulated cells was only 0.15 ± 0.20 pmol cAMP/10^6 cells, $n = 5$. Tested alone, none of the AC activators at the concentrations tested, histamine (30 μM), salbutamol (10 μM) and forskolin (10 μM), produced a significant net increase in intracellular cAMP levels, $n = 4$. In the absence of an AC activator, the

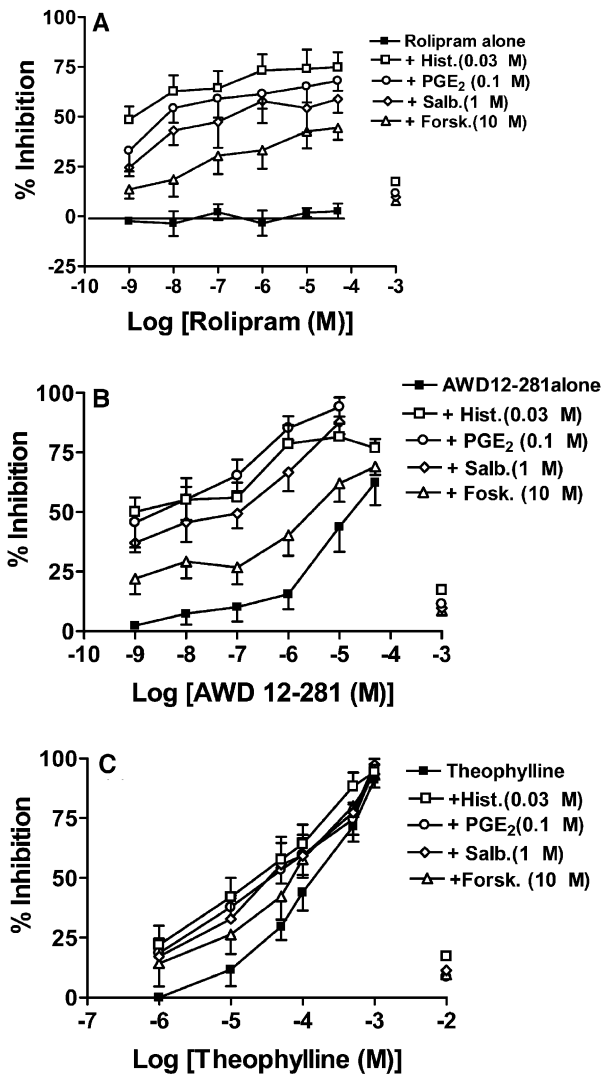

Fig. 4. The effect of the adenylate cyclase (AC) activators (histamine, prostaglandin E_2, salbutamol and forskolin) on the ability of rolipram (A), AWD 12-281 (B) and theophylline (C) to inhibit EPO release from human eosinophils induced by complement fragment C5a (10 nM). The uninhibited release was in the range 13 to 45% of the total cell content. Cells were first pre-incubated with the PDE inhibitors for 10 min, followed by the AC activators for another 10 min before stimulation. Values are means ± S.D., $n = 6$ or 7. The unconnected symbols represent the effect of the AC activators alone.

Table 1
The interaction of PDE inhibitors and adenylate cyclase activators in the elevation of intracellular cAMP in human eosinophils
ND = not done.

	Net increase in intracellular cAMP (pmol/10^6 eosinophils)			
	+ Buffer, $n = 5$	+ Histamine (30 μM), $n = 4$	+ Salbutamol (10 μM), $n = 4$	+ Forskolin (10 μM), $n = 4$
Buffer	[0.15 ± 0.20]	0.10 ± 0.15	0.06 ± 0.10	0.16 ± 0.21
Rolipram (10 μM)	0.24 ± 0.17	1.46 ± 0.38[a]	0.62 ± 0.29	1.77 ± 0.30[a]
Rolipram (30 μM)	0.48 ± 0.27[b]	2.00 ± 0.33[a]	1.54 ± 0.40[a]	2.63 ± 0.45[c]
AWD 12–218 (10 μM)	0.25 ± 0.10	1.60 ± 0.44[a]	1.20 ± 0.35[a]	1.56 ± 0.37[a]
AWD 12–281 (30 μM)	0.40 ± 0.21[b]	1.73 ± 0.27[a]	1.67 ± 0.38[a]	2.36 ± 0.48[a]
Theophylline (300 μM)	0.15 ± 0.24	0.88 ± 0.36[a]	ND	1.06 ± 0.24[a]
Theophylline (1000 μM)	0.32 ± 0.18	2.12 ± 0.48[a]	ND	ND

[a] $P < 0.05$ vs. the expected sum of values for PDE inhibitor alone and AC alone.
[b] $P < 0.05$ vs. buffer alone.
[c] $P < 0.01$ vs. the expected sum of values for PDE inhibitor alone and AC activator alone.

PDE inhibitors produced small dose-dependent increases in cAMP that reached statistical significance only at the higher concentrations of rolipram (30 μM) and AWD 12-281 (30 μM). However, when combined with the AC activators, synergistic increases in cAMP responses were seen for both the selective PDE IV inhibitors—rolipram and AWD 12-281—and the non-selective PDE inhibitor—theophylline. For example, in the absence of an AC activator, rolipram (30 μM) produced a net increase of 0.48 ± 0.27 pmol cAMP/10^6 cells, whereas in the absence of rolipram, the net increase produced by the AC activators was 0.10 ± 0.15, 0.06 ± 0.10 and 0.16 ± 0.21 pmol cAMP/10^6 for histamine (30 μM), salbutamol (10 μM), and forskolin (10 μM), respectively. When rolipram was combined with these AC activators, the net increase was 2.00 ± 0.33, 1.54 ± 0.40 and 2.63 ± 0.45 pmol/10^6 cells, respectively. The differences between these values and the expected sums of the individual values for rolipram and the corresponding AC activators were statistically significant, $P < 0.05$–0.01, thus indicating synergism.

It is to be noted that, in the absence of an AC activator, even the highest concentration of theophylline (1000 μM) (which inhibited EPO release by > 90%) failed to produce a significant increase in intracellular cAMP.

4. Discussion

In this study, four highly potent and selective PDE IV inhibitors [rolipram, AWD 12-281, RP 73401 and SB 207499) were used to further investigate the differential effect of selective and non-selective PDE inhibitors, with special reference to human eosinophil degranulation and the role of intracellular cAMP. The results demonstrated that while all four PDE IV inhibitors were effective in inhibiting C5a-induced O_2^- release, none, except AWD 12-281, had any effect on EPO release. In contrast, the non-selective inhibitors, theophylline and IBMX were effective in inhibiting both responses. For the inhibition of O_2^- release, the PDE IV inhibitors were, however, two to three orders of magnitude more potent than the non-selective ones, though with comparable efficacy. These results are in agreement with those previously reported for the respiratory burst in eosinophils of both guinea pigs (Dent et al., 1991; Souness et al., 1991, 1995) and humans (Cohan et al., 1996; Dent et al., 1994; Ezeamuzie and Al-Hage, 1998) as well as for degranulation of human eosinophils (Hatzelmann et al., 1995). In human whole blood, rolipram was reported to inhibit platelet activating factor-induced up-regulation of adhesion molecules in eosinophils (Berends et al., 1997). This agrees with the present result with respect to O_2^- release but not degranulation, and thus supports the view that different responses of eosinophils can be differentially affected by drugs (Ezeamuzie and Nwankwoala, 1997).

The combined inhibitory effect of PDE IV inhibitors and the AC activators on eosinophil degranulation was mostly synergistic, and this was reflected in the corresponding synergistic increase in the intracellular levels of cAMP. For theophylline, however, there was a synergistic increase in intracellular cAMP, but only a marginal increase in the inhibition of degranulation (additive at most). This may suggest that the non-selective PDE inhibitors generally require lower intracellular levels of cAMP compared with selective ones, in order to attenuate this response or that the non-selective inhibitors are somehow able to inhibit degranulation in an essentially cAMP-independent manner.

The finding that AWD 12-281 can inhibit eosinophil degranulation is quite interesting, and perhaps makes the drug the first PDE IV inhibitor to possess this property. It is unlikely that this unique effect of AWD 12-281 is related to its potency since the drug is comparable in potency to rolipram and SB 207499 and at least 10 times

less potent than RP 73401, in both cell-free PDE IV isoenzyme inhibition and inhibition of pro-inflammatory cell responses (Cohan et al., 1996; Barnette et al., 1998; Heer et al.,1999). Furthermore, the fact that AWD 12-281, synergized with AC activators, causes inhibition of degranulation even at concentrations of AWD 12-281 that were effective on their own, suggests that the inhibitory activity may be unrelated to the PDE IV inhibitory activity of the drug.

In addressing the reason why the non-selective PDE inhibitors, but not the selective PDE inhibitors, inhibited eosinophil degranulation, Hatzelmann et al. (1995) showed that rolipram became an effective inhibitor in the presence of the β_2-adrenoceptor agonist salbutamol. A similar interaction was also reported for the inhibition of zymosan-induced interleukin-8 release from human neutrophils (Au et al., 1998) and O_2^- release from human eosinophils (Dent et al., 1994). These observations would suggest that selective PDE IV inhibitors might require an additional cAMP signal to be effective. The present study confirms such findings and demonstrates that such signals could be generated via various AC-linked receptors (histamine H_2-receptors, prostaglandin E receptors and β_2-adrenoceptors), as well as via direct AC activation (forskolin). Furthermore, by actually measuring the cAMP response elicited by the PDE IV inhibitors alone and in combination with histamine, salbutamol or forskolin, the present study has provided direct evidence that synergistic cAMP generation was indeed the additional signal required. The limitation imposed by the large number of eosinophils required in these experiments did not allow us to test prostaglandin E_2.

Why the non-selective PDE inhibitors do not require this extra cAMP signal to inhibit degranulation is unclear. It is unlikely that the inhibition of more than one PDE isoenzyme is necessary for degranulation to be attenuated. This is because the human eosinophil is known to contain predominantly the PDE IV isoenzyme (Torphy and Undem, 1991; Giembycz, 1992), and also because the highly selective PDE IV inhibitor AWD 12-281 was effective. One possibility is that the non-selective PDE inhibitors may, in addition to PDE inhibition, also somehow activate AC or directly activate some cAMP-dependent protein kinases, as has been previously suggested (Parsons et al., 1988; Tomes et al., 1993).

Although the synergistic generation of extra cAMP appears to be the basis for the anti-degranulatory effectiveness of PDE IV inhibitors in the presence of AC activators, a closer look at the relationship between the level of intracellular cAMP generated and the extent of inhibition revealed a number of inconsistencies. Firstly, forskolin (10 μM), which synergized relatively more effectively with rolipram than the receptor-mediated AC activators in raising intracellular cAMP, was the least effective in potentiating the anti-degranulatory effect of rolipram. Secondly, at the concentration of 30 μM, both rolipram and AWD 12-281 produced comparable cAMP responses, whereas only AWD 12-281 was effective in inhibiting degranulation in the absence of AC activators. Thirdly, in the absence of PDE inhibitors, histamine and, to a lesser extent, prostaglandin E_2 (at concentrations above 0.1 μM) were able to inhibit eosinophil degranulation, but under the same conditions, they were incapable of significantly elevating intracellular cAMP.

The reason for these discrepancies is uncertain, but it highlights the complex nature of the role of cAMP in the regulation of eosinophil responses. There is, of course, the possibility that cAMP generated by different agents or under different circumstances may be qualitatively different or differentially used by different sub-populations of cAMP-dependent protein kinases. No evidence is yet available to support these possibilities. Further studies are on-going to further clarify the exact role of cAMP in the regulation of eosinophil functions.

The PDE IV inhibitors used in this study are all experimental drugs for which therapeutic concentrations have yet to be established. However, given that the synergistic interactions of these compounds with AC activators occurred even at very low concentrations of the PDE inhibitors (as low as 1 nM), it is likely that such interactions will be therapeutically relevant. For theophylline, the essentially additive interaction with the AC activators was also quite apparent in its therapeutic concentration range of 10–100 μM. These interactions are, therefore, expected to contribute to the overall anti-inflammatory potency of these drugs in vivo. For example, in the bronchial inflammation that underlines asthma, the concentrations of the endogenous AC-activating autocoids, such as histamine and prostaglandin E_2, are bound to be raised. In such a scenario, the resulting potentiation of the action of PDE IV inhibitors might in fact be a major contributing factor to the anti-inflammatory and anti-asthma potency of these drugs.

In summary, this study has shown that, unlike the non-selective PDE inhibitors, selective PDE IV inhibitors require an additional cAMP signal to be able to inhibit eosinophil degranulation. This signal can be generated by both direct and receptor-mediated AC activation. The exact cause–effect relationship between intracellular cAMP and inhibition of degranulation is, however, not a simple one, hence, more studies are required to clarify the role of cAMP in the regulation of human eosinophil responses. Nevertheless, the potentiation of the inhibitory action of PDE IV inhibitors by endogenous autacoids is likely to be important in the overall in vivo anti-inflammatory action of these drugs.

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