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Adenosine A_3 receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release

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1 The role of adenosine A_3 receptors on human eosinophil degranulation and superoxide anion (O_2^-) release was studied *in vitro* using the complement fragment C5a as the main stimulus and employing a number of selective agonists and antagonists.

2 In the presence of cytochalasin B (CB), C5a induced a dose-dependent release of the granular eosinophil peroxidase (EPO), but not O_2^-, whereas in the absence of CB O_2^-, but not EPO, was released.

3 C5a-induced EPO release was inhibited dose-dependently by the selective A_3 agonist N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine (IB-MECA) and to a lesser extent by the less-selective N^6-2-(4-amino-3-iodophenyl) ethyladenosine (APNEA). The IC_{50} (95% CI) for IB-MECA was 6.8 μM (3.1–12.0 μM). At concentrations up to 100 μM, neither adenosine nor the selective A_1 agonist N-cyclopentyladenosine (CPA) and the selective A_2 agonist 2-[[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-*N*-ethylcarboxamidoadenosine (CGS 21680) had any significant effect.

4 The inhibitory effect of IB-MECA was almost completely abolished by pre-treatment with 1 μM of the selective A_3 antagonist 9-chloro-2-(2-furyl)-5-phenylactylamino[1,2,4]triazolo[1,5-c]quinazoline (MRS 1220), but not the selective A_1 antagonist 1,3-dipropyly-8-cyclopentylxanthine (DPCPX) or the selective A_2 antagonist 3,7-dimethyl-1-propargylxanthine (DMPX).

5 IB-MECA also significantly inhibited C5a-induced O_2^- release with IC_{50} (95% CI) of 9.5 μM (4.6–13.1 μM) whereas adenosine and the A_1 agonist CPA potentiated this effect at low concentrations. The potentiation appeared to be a result of their direct O_2^- release from these cells, probably mediated *via* A_1 receptors. The inhibition by IB-MECA was selectively reversed by MRS 1220.

6 These results show that the A_3 receptors on human eosinophils mediate inhibition of both degranulation and O_2^- release and suggest a therapeutic potential for A_3 agonists in diseases such as asthma in which activated eosinophils are involved.

Keywords: Adenosine; A_3 receptors; eosinophils; superoxide anion; degranulation

Abbreviations: APNEA, N^6-2-(4-amino-3-iodophenyl) ethyladenosine; CB, Cytochalasin B; CGS21680, 2-[[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-*N*-ethylcarboxamidoadenosine; CPA, N-cyclopentyladenosine; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 1,3-dipropyly-8-cyclopentylxanthine; EPO, Eosinophil peroxidase; FMLP, N-formyl methyl-leucyl-phenylalanine; IB-MECA, N^6-(3-iodobenzyl)-5′*N*-methylcarbamoyladenosine; MRS 1220, 9-chloro-2-(2-furyl)-5-phenylactylamino[1,2,4]triazolo[1,5-c]quinazoline; PAF; platelet activating factor; PMA, phorbol myristate acetate

Introduction

The potential role of adenosine as a modulator of allergic reactions, especially asthma, has been a subject of growing interest. Pre-incubation of rat mast cells with adenosine enhances IgE-dependent release of allergic mediators (Church *et al*., 1986; Ramkumar *et al*., 1993). In asthmatics, there is a significantly raised concentration of adenosine in the bronchoalveolar lavage fluid compared to normal subjects (Driver *et al*., 1993), and the inhalation of adenosine has been reported to cause bronchoconstriction in asthmatics, but not normal subjects (Church & Holgate, 1993). Furthermore, isolated human bronchial smooth muscles have been shown to be hyperresponsive to adenosine (Bjorck *et al*., 1992). In these studies, the effects were significantly blocked with histamine antagonists and leukotriene antagonists, thus suggesting that they were due to the release of allergic mediators from lung mast cells.

The existence of at least four distinct G-protein-coupled receptors A_1, A_{2A}, A_{2B} and A_3 which mediate the actions of adenosine has been confirmed by both functional characterization and gene cloning (Furlong *et al*., 1992; Sajjadi & Firestein, 1993; Gurden *et al*., 1993; Fredholm *et al*., 1994; Palmer & Stiles, 1995; Murrison *et al*., 1996). In rats, the recently characterized A_3 receptors are now believed to mediate direct mast cell degranulation (Fozard *et al*., 1996) or enhancement of allergen-induced mediator release (Ramkumer *et al*., 1993). In man, A_3 receptors are richly expressed in the lungs (Salvatore *et al*., 1993) and most of these appear to be present on eosinophils rather than mast cells (Bai *et al*., 1994; Kohno *et al*., 1996; Walker *et al*., 1997). This has been confirmed on isolated blood eosinophils by both receptor mRNA transcript analysis and ligand binding (Kohno *et al*., 1996).

Eosinophils are known to play important roles in the pathophysiology of allergic diseases, especially asthma (Frigas & Gleich, 1986; Barnes, 1989). They infiltrate the asthmatic lung where they release tissue-damaging granular proteins such as the major basic protein (MBP), allergic mediators such as

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the leukotrienes, as well as oxygen free radicals, which in concert orchestrate bronchial inflammation and consequent bronchial hyperresponsiveness.

Few studies have investigated the functional role of A_3 receptors on human eosinophils. Kohno *et al*. (1996) have reported A_3-mediated elevation of intracellular calcium in these cells – a finding that is in agreement with the stimulatory role of this receptor subtype on rat mast cells. However, others have reported inhibition of human eosinophil migration induced by platelet activating factor and other soluble mediators (Walker *et al*., 1997; Knight *et al*., 1997).

The objective of the current study was to clarify the functional role of A_3 receptors on human eosinophils based on two major responses – degranulation and superoxide anion release, employing a number of selective adenosine receptor agonists and antagonists.

Methods

Isolation of blood eosinophils

Fresh blood was obtained from consenting healthy or mildly atopic adults who have taken no medications in the last 72 h. 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 centrifuged at $200 \times g$ for 10 min at room temperature. After aspirating off 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 2-step percoll gradient (1.080 and 1.090 g ml^{-1}) and centrifuged at $900 \times g$ on 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 a hypotonic lysis of contaminating erythrocytes with ice-cold distilled water, and readjusting 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 the 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 after the final wash, the cells were pelleted by centrifugation and then resuspended in 500 μl of prewashed immunomagnetic beads pre-coated with affinity purified sheep anti-mouse IgG (2×10^8 coated beads) and incubated in ice for 1 h with frequent tube rotation. The immunomagnetically-labelled 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 count 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%.

Eosinophil peroxidase (EPO) release

Purified eosinophils were used at a concentration of 5×10^5 cells ml^{-1}. 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 (CB) was added and after 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, 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 (OPD) 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 PBS-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 plate read at 490 nm. Values were expressed as percentage of 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 30 min incubation, but usually lower with more prolonged incubation.

Superoxide anion release

Superoxide anion (O_2^-) generation was determined by the superoxide dismutase (SOD)-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 dispensed into each well of the 96-well microplate containing 50 μl of cytochrome c (100 μM, final) and 50 μl reaction buffer. After pre-warming the mixture, 50 μl of the stimulus was then added and the mixture (total of 200 μl) 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 ($4 \times$ required concentration in reaction buffer) was added in place of the buffer and incubated with the cells for 5 min before the addition of the stimulus. In some experiments in which the reversal of the effect of the drugs was attempted, the reversing agent was added as 5 μl of a 40 fold required concentration. After incubation of the reaction mixtures, 150 μl was transferred to a fresh plate and the absorbance read at 550 nm on the Titertek Multiscan (Flow Labs, Rickmansworth, U.K.) plate reader. The amount of O_2^- generated was estimated as nanomoles of ferricytochrome c reduced per 10^6 cells per hour using the extinction coefficient of 2.1×10^{-4} M^{-1} cm^{-1}.

Chemicals and biochemical reagents

The following reagents and materials were purchased from Sigma Chemical Co., St. Louis, U.S.A.: recombinant human C5a, FMLP, percoll, ferricytochrome c (from horse heart), (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid])(-HEPES), SOD (from bovine erythrocytes), bovine serum albumin (BSA), OPD, dimethylsulphoxide (DMSO) and cytochalasin B. Adenosine, N^6-2-(4-amino-3-iodophenyl) ethy-

ladenosine (APNEA), N-cyclopentyladenosine (CPA), 3,7-dimethyl-1-propargylxanthine (DMPX) 9-chloro-2-(2-furyl)-5-phenylactylamino[1,2,4]triazolo[1,5-c]quinazoline (MRS 1220) and 8(-*p*-sulfophenyl)-theophylline (8-pSPT) were obtained from Research Biochemicals Corp., Natick, MA, U.S.A. N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine (IB-MECA), 2-[[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-*N*-ethylcarboxamidoadenosine (CGS 21680) and 1,3-dipropyly-8-cyclopentylxanthine (DPCPX) were purchased from Tocris Cookson Ltd, Bristol, U.K. Mouse monoclonal anti-human CD16 antibody (clone FcR gran1) was obtained from CLB, Amsterdam, Netherlands, while the magnetic beads (coated with sheep anti-mouse IgG) were supplied by Dynal AS, Oslo, Norway. All the inorganic salts were obtained from British Drug House (BDH) and were all of analytical grades.

Stock solutions of all adenosine receptor agonists and antagonists were made in DMSO to concentrations in the range ($1-4\times10^{-1}$ M) and then diluted directly in buffer. The final concentration of DMSO present 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 down in reaction buffer.

Statistical analysis

Experimental data are presented as mean ± standard deviation from the number (*n*) of independent experiments. The drug concentrations producing 50% inhibition of response (IC_{50} values) were calculated using the concentration-effect curves by non-linear regression analysis using GraphPad InPlot (Graph*i*Pad Software Inc., Philadelphia, U.S.A.). Statistical significance (*P*) was determined by the unpaired *t*-test and analysis of variance (ANOVA) as appropriate (InStat, Graph*i*Pad, Software Inc. U.S.A.).

Results

C5a-induced eosinophil degranulation and O_2^- release

As shown in Figure 1a, in the presence of CB, C5a produced a concentration-dependent release of the granular EPO from purified human eosinophils. The release generally began at around 10^{-9} M, and at the highest concentration tested, 10^{-7} M, a net EPO release of 33.8 ± 4.4% of the cell content was obtained. No EPO release occurred in the absence of CB. In contrast, C5a-induced O_2^- release occurred in the absence, but not presence of CB. The release occurred in the same concentration range as EPO, but with a clear peak at 10^{-8} M, at which concentration O_2^- release equivalent to the amount reducing 43.1 nm cytochrome c/10^6 cells h^{-1} was obtained, Figure 1b.

Effect of adenosine receptor agonists on EPO release

Pre-incubation of eosinophils with the selective A_3 agonist IB-MECA caused a pronounced and concentration-dependent inhibition of C5a-induced EPO release, Figure 2. The IC_{50} (95% CI) was 6.8×10^{-6} M (3.1×10^{-6} M – 1.2×10^{-5} M) and at the highest concentration tested, (10^{-4} M), the percentage inhibition was 72.5 ± 6.4%. APNEA, which is a less-selective A_3 agonist than IB-MECA produced a smaller, but statistically significant, inhibition with the highest concentration, tested (10^{-4} M) producing only 47.3 ± 8.8% inhibition. Neither adenosine, nor the selective A_1 agonist CPA or the selective A_2 agonist CGS 21680 produced any statistically significant inhibition, although at high concentrations (10^{-5} M – 10^{-4} M) adenosine and CGS 21680 consistently produced a small inhibition (10 – 15%). A similar inhibitory effect by IB-MECA and APNEA was also seen when EPO release was induced by N-formlymethionyl-leucyl-phenylalanine (FMLP) in the presence of CB (data not shown). At the concentrations tested, none of these agonists had any significant effect of their own on EPO release, cell viability, peroxidase activity or EPO recovery.

The effect of selective antagonists at various adenosine receptors on IB-MECA-induced inhibition is shown in Figure 3. The pre-incubation of the cells with 10^{-6} M of the recently available selective A_3 antagonist – MRS 1220 (Kim *et al*., 1996; Jacobson *et al*., 1997) for 5 min before the addition of IB-MECA, resulted in almost completely reversal of the inhibition as seen by the pronounced depression of the concentration-inhibition curve of the latter, Figure 3b. At the same concentration, neither the selective A_1 antagonist DPCPX nor the selective A_2 antagonist DMPX or the non-selective A_1/A_2 antagonist 8-pSPT produced any significant reversal of IB-MECA-induced inhibition (Figure 3a, c and d). At the concentration used, the antagonists neither induced EPO release by themselves nor affected C5a-induced EPO release, peroxidase activity or EPO recovery. The use of a single moderate concentration of the antagonists was deliberate since at high concentrations of agonists and antagonists, the combined solvent (DMSO) concentration may exceed the 0.05%, which is inhibitory to eosinophils.

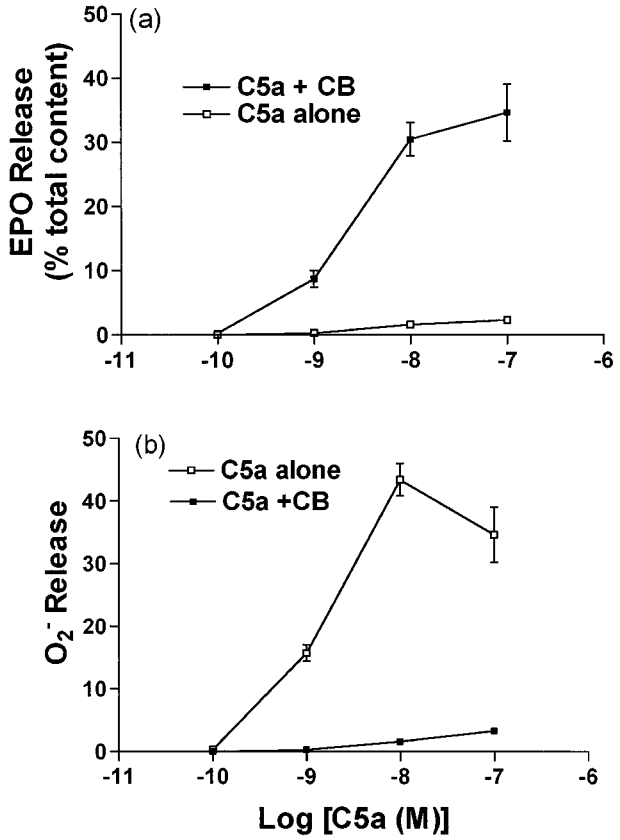

Figure 1 C5a-induced release of EPO (a) and O_2^- (b) from human eosinophils in the presence and absence of cytochalasin B (CB). The O_2^- release is expressed as nm cytochrome c reduced/10^6 cells h^{-1}. Values are mean ± s.d. for eight experiments.

Effect of adenosine receptor agonists on O_2^- release

In contrast to the lack of a direct EPO-releasing effect of the adenosine receptor agonists, these same agonists were found to directly induce some O_2^- release. As shown in Figure 4a and b, the releases which were concentration-dependent and generally peaked in the range 10^{-7} M – 10^{-6} M, were moderate in magnitude (10 – 25 nm cytochrome c reduced 10^6 cells h^{-1}). Nevertheless, the peak releases were statistically significant ($P<0.05$, $n=5$) for adenosine, CPA and APNEA, but not for CGS 21680 and IB-MECA. As shown in Figure 5, pre-incubation of eosinophils with low concentrations of all these agonists augmented C5a-induced O_2^- generation in an apparently additive manner. At higher concentrations (10^{-5} M – 10^{-4} M), however, all the agonists produced some inhibitions, but these were statistically significant only for APNEA and IB-MECA. At 10^{-4} M the inhibitions were 44.6 ± 7.6 and 73.4 ± 8.1%, respectively, for APNEA and IB-MECA. The IC_{50} value (95% CI) for the latter was 9.5×10^{-6} M (4.6×10^{-6} – 1.3×10^{-5} M). Similar inhibitory effects were seen with platelet activating factor (PAF)-induced, but not phorbol myristate acetate (PMA)-induced O_2^- generation (data not shown).

To determine whether the O_2^- release enhancing and inhibiting effects of these agonists were mediated *via* adenosine receptors, the effect of selective antagonists was studied. As shown in Table 1, at the concentration of 10^{-6} M, the enhancements of C5a-induced O_2^- release by CPA and APNEA were significantly reversed by the selective A_1 antagonist DPCPX, but not the selective A_2 antagonist DMPX or the selective A_3 antagonist MRS 1220. On the other hand, at the same concentration, MRS 1220, but not DPCPX or DMPX significantly reversed the inhibition produced by 10^{-5} M IB-MECA. These results suggest that the enhancement of C5a-induced O_2^- release (most probably *via* direct O_2^- release) is mediated through the A_1 receptors whereas the suppression was mediated *via* the A_3 receptors. None of the agonists and antagonists had any significant superoxide dismutase activity at the concentrations tested, as assessed by the xanthine/xanthine oxidase system.

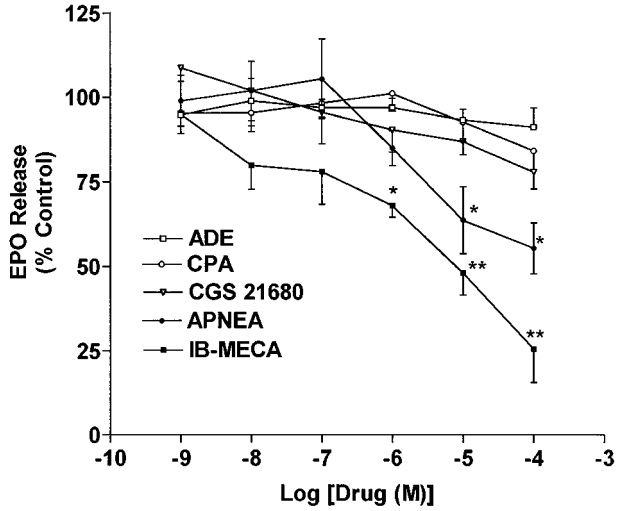

Figure 2 The effect of adenosine receptor agonists (adenosine, CPA, CGS 21680, APNEA and IB-MECA) on EPO release from human eosinophils induced by 10^{-8} M C5a in the presence of CB. Uninhibited release was in the range 22 – 35% of the cell content. Values are means ± s.d. for 5 – 7 experiments. $^*P<0.05$; $^{**}P<0.01$.

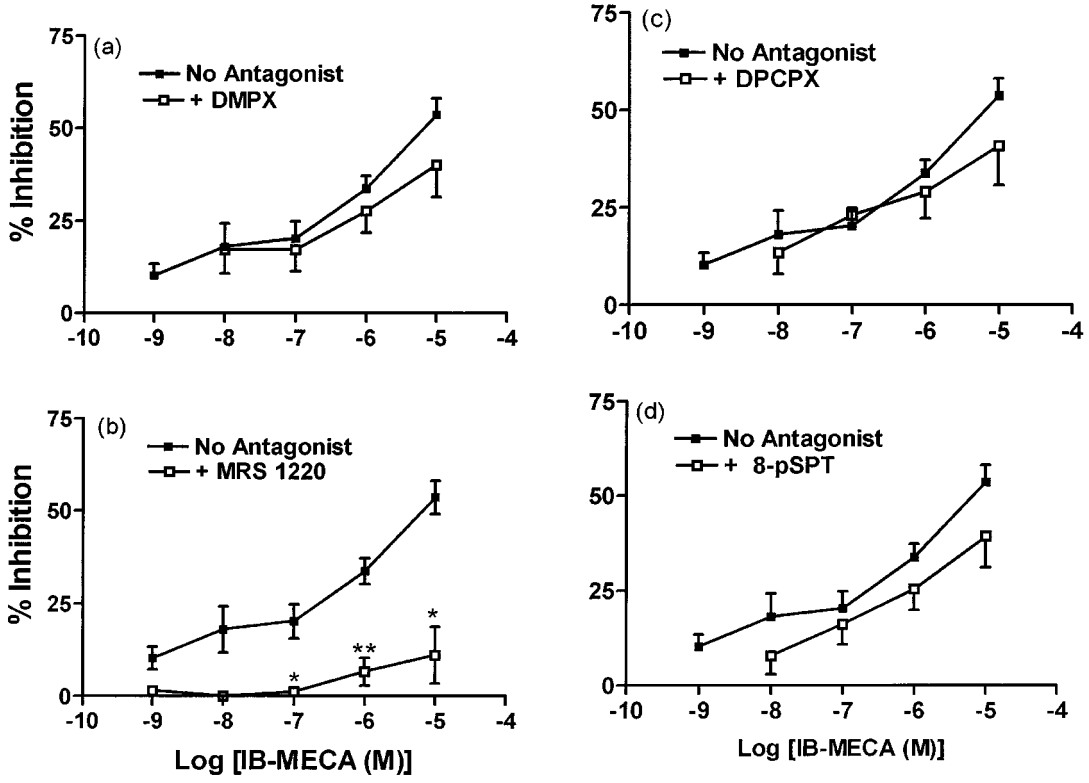

Figure 3 Reversal of IB-MECA-induced inhibition of EPO release by adenosine receptor subclass antagonists – DPMX (a), MRS 1220 (b), DPCPX (c) and 8-pSPT (d). Cells were first incubated with the antagonist for 5 min, followed by IB-MECA, and after a further 5 min stimulated with 10^{-8} M C5a in the presence of CB. Values are means ± s.d., $n=5$. $^*P<0.05$; $^{**}P<0.01$.

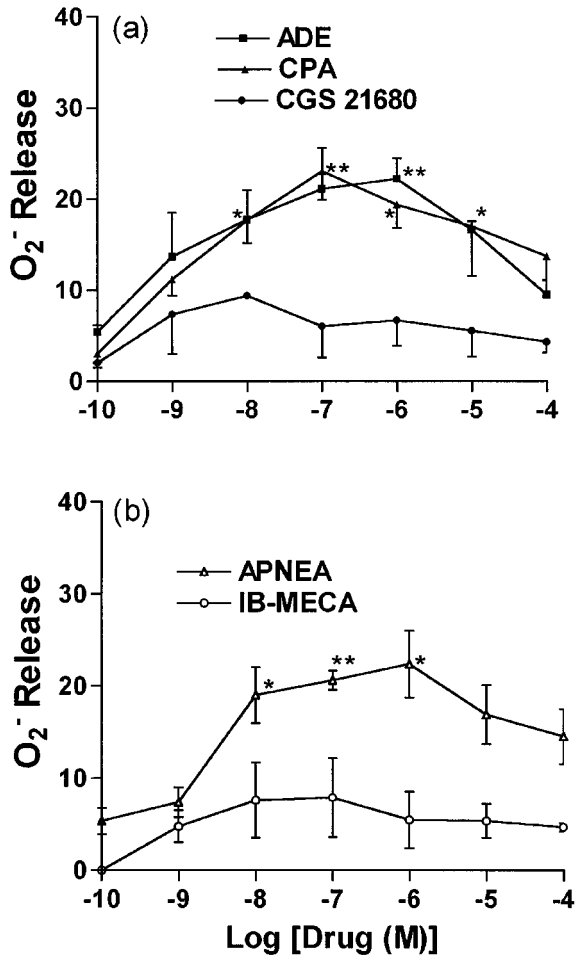

Figure 4 Direct release of O_2^- from human eosinophils by adenosine receptor agonists–adenosine, CPA and CGS 21680 (a); APNEA and IB-MECA (b). The O_2^- release is expressed as nm cytochrome c reduced/10^6 cells h^{-1}. Values are means±s.d., $n=7$. *$P<0.05$; **$P<0.01$.

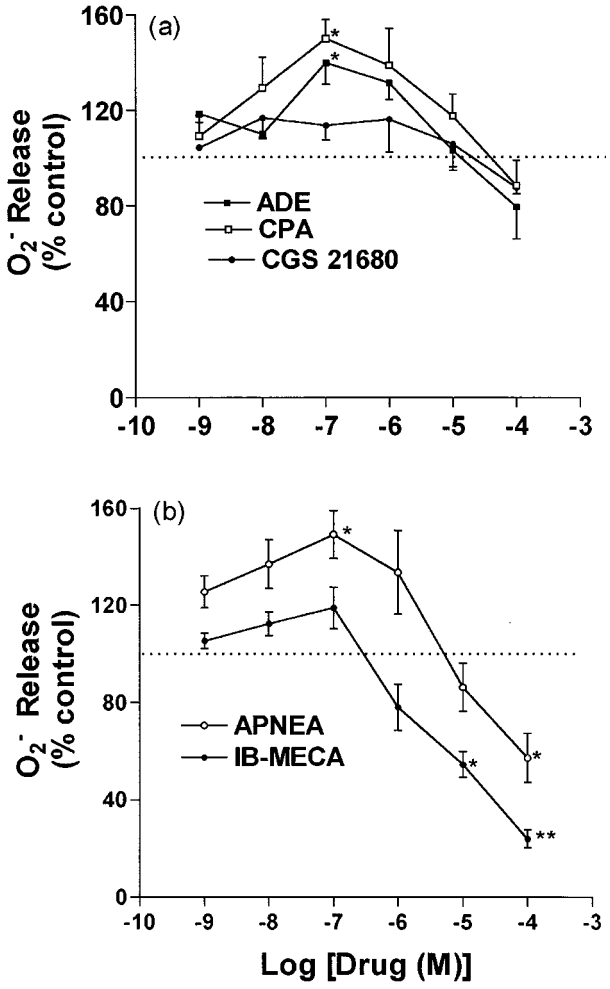

Figure 5 The effect of adenosine receptor agonists–adenosine, CPA, and CGS (a), and APNEA and IB-MECA (b) on the O_2^- release induced by C5a (10^{-8} M). The O_2^- release by C5a alone was in the range 30–45 nm cytochrome c reduced/10^6 cells h^{-1}. Values are means±s.d., $n=5$. *$P<0.05$; **$P<0.01$.

Table 1 The effect of adenosine receptor agonists on C5a-induced O_2^- release from human eosinophils and its reversal by selective antagonists

		Superoxide anion release (nm cytochrome c reduced/10^6 cells h^{-1}) + C5a (10^{-8} M)			
		Buffer	*DPCPX* (10^{-6} M)	*DMPX* (10^{-6} M)	*MRS1220* (10^{-6} M)
Buffer		42.2±7.5	45.6±6.1	37.1±12.7	49.8±8.0
CPA	(10^{-7} M)	64.5±9.2**	43.9±9.3†	59.2±7.6	67.4±9.7
APNEA	(10^{-7} M)	57.2±7.9*	35.4±5.6†	46.3±5.5	52.8±10.5
IB-MECA	(10^{-5} M)	26.8±6.2**	29.4±5.0*	19.7±7.7*	40.9±5.9†

*$P<0.05$, **$P<0.01$ with respect to C5a alone; †$P<0.01$ with respect to C5a + agonist, $n=5$.

Discussion

The recent confirmation of a high level of expression of A_3 receptors on human eosinophils (Bai *et al*., 1994; Kohno *et al*., 1996; Walker *et al*., 1997), has subsequently raised the question of the functional roles of these receptors. In the present study, we have shown that these receptors mediate two important anti-inflammatory functions of human eosinophils – the inhibition of degranulation and inhibition of oxygen radicals generation. This conclusion was based on the following facts. Firstly, the inhibition of both responses was induced by the selective A_3 agonist IB-MECA, and to a lesser extent by the relatively less-selective A_3 agonist, APNEA (Fozard & Carruthers, 1993), but not the selective A_1 and A_2 agonists (CPA and CGS 21680, respectively). Secondly, these inhibitory effects were significantly reversed by moderate concentrations of the selective A_3 receptor antagonist MRS 1220, but not the selective A_1 and A_2 receptor antagonists (DPCPX and DMPX,

respectively). Although this report is based on the use of the complement fragment C5a as the stimulus, we observed the same effects when FMLP (in the presence of CB) was employed as the degranulation stimulus. This rules out the possibility that IB-MECA was acting by interfering with C5a binding to its receptors.

Rather surprisingly, we noted that relatively low concentrations of adenosine and the selective A_1 agonist CPA, as well as APNEA, but not the selective A_2 and A_3 agonists (CGS 21680 and IB-MECA, respectively), were able to directly induce significant O_2^- release from eosinophils. Since this effect was reversed by the A_1, but not the A_2 and A_3 receptor antagonists, it suggests that the effect was A_1-mediated. The fact that APNEA, but not IB-MECA, possesses the direct O_2^- releasing effect is consistent with the former possessing a lower A_1/A_3 selectivity (Fozard & Carruthers, 1993). At higher concentrations, however, both A_3 agonists produced significant inhibition of C5a-induced O_2^- release. The observation that PAF-induced, but not PMA-induced, O_2^- generation, was inhibited by these A_3 agonists supports the view that the agents were acting *via* membrane receptors. The selective reversibility of this inhibition by the selective A_3 receptor antagonist MRS 1220, confirms it as an A_3 receptor-mediated event. Taken together, these effects suggest that the activity of human eosinophil NADPH oxidase system is dually regulated by adenosine receptors – being enhanced and suppressed *via* the A_1 and A_3 receptors, respectively.

The inhibitory role of A_3 receptors, as the present results show, is in agreement with the two recent reports showing that A_3 receptor activation inhibited human eosinophil chemotaxis induced by a number of endogenous chemoattractants (Knight *et al*., 1997; Walker *et al*., 1997). Furthermore, Bouma *et al*. (1997), have obtained a similar result in lipopolysaccharide- and transforming growth factor-induced neutrophil degranulation, although in this study, A_2 receptors also appeared to contribute. On the other hand, Kohno *et al*. (1996), had earlier reported that A_3 receptor activation resulted in a transient increase in the intracellular Ca^{2+} in human eosinophils. Although we did not measure changes in Ca^{2+} levels in this study, it is well established that increased intracellular Ca^{2+} is an important requirement for eosinophil activation induced by C5a and other agonists such as PAF, RANTES and eotaxin (Van der Bruggen & Koenderman, 1996; Elsner *et al*., 1996). A similar contradiction is also apparent by virtue of the second messenger system reportedly utilized by the A_3 receptor subtype. The activation of this receptor is known to lead to the activation of phospholipase C and D (Ali *et al*., 1990) and to the inhibition of adenylate cyclase (Zhou *et al*., 1992). Both of these effects favour eosinophil activation because activated PLC results in the generation of inositol triphosphate (IP_3) and the consequent mobilization of intracellular Ca^{2+} while the inhibition of adenylate cyclase prevents a rise in intracellular concentrations of cyclic AMP – a powerful inhibitor of eosinophil functions (Kita *et al*., 1991). One possible explanation for this anomaly is that the effect of A_3-mediated mobilization of internal Ca^{2+} *via* IP_3 generation is overridden by the activation of PKC *via* diacylgycerol, since PKC is known to negatively regulate EPO and leukotrienes release, though not respiratory burst, in human eosinophils (Kernen *et al*., 1991; Grix *et al*., 1996). Whether A_3 receptors on human eosinophils actually utilize these pathways is of course yet to be determined. On the other hand, it is of course possible that the reported Ca^{2+} response merely reflected the rather activated or primed status of eosinophils from hypereosinophilic or overtly allergic patients as employed in that study. Meanwhile, the mechanism of A_3 mediated inhibition of eosinophil functions remains unknown at the moment and deserves serious investigation.

The reason for the failure of adenosine itself to significantly inhibit these responses is not clear, but may probably reflect its uptake and metabolism. This is, however, difficult to reconcile with the fact that relatively low concentrations of adenosine significantly enhanced O_2^- release. Dipyridamole – a nucleoside uptake inhibitor – could not be used because it caused a small enhancement of EPO release by itself.

The intriguing opposing effects of CB on EPO and O_2^- releases by human eosinophils have been previously reported (Zeck-Kapp *et al*., 1995). It has been suggested that CB which is a microtuble depolarizer, enhances degranulation by removing the cytoskeletal barriers that restrict access of cytoplasmic granules to the plasma membrane in the process of exocytosis (Burgoyne, 1990). It is, therefore, conceivable that such effect may, at the same time, block the translocation of components of the O_2^- generating NADP oxidase to the plasma membrane – a process that is dependent on viable microfilaments.

The *in vivo* relevance of the A_3 receptors in man is currently uncertain. In man, A_3 receptors are richly expressed in the lungs (Salvatore *et al*., 1993) but these appear to be present on eosinophils, but not mast cells (Bai *et al*., 1994; Walker *et al*., 1997). In asthmatics, the concentration of adenosine in the bronchoalveolar lavage fluid is higher than in normal subjects (Driver *et al*., 1993), and the inhalation of adenosine causes bronchoconstriction in asthmatics, but not normal subjects (Church & Holgate, 1993). Since this effect could be significantly blocked by histamine antagonists, it is believed to be due to the release of allergic mediators from mast cells. Whether these effects of adenosine in asthmatics are due to a pathologically-induced expression of A_3 receptors on bronchial mast cells is yet to be determined. Although at the highest concentration used (10^{-4} M) adenosine itself had only a small inhibitory effect on eosinophil functions, it is possible that the high concentration found in asthmatic lungs (Driver *et al*., 1993), may be sufficient to produce significant inhibition. In that case, the presence of high concentrations of adenosine in the asthmatic lungs may represent a strategy for dampening allergic eosinophilic inflammation *via* eosinophil A_3 receptor activation.

In contrast to the uncertainty of the physiological roles of the endogenous adenosine *vis-à-vis* eosinophil A_3 receptors, the suppressive effect of selective A_3 receptor agonists on eosinophil functions is of considerable therapeutic interest. Eosinophils are the major cell type involved in the pathophysiology of allergic diseases including asthma (Frigas & Gleich, 1986; Barnes, 1989). Their infiltration and subsequent release of toxic granule proteins and oxygen radicals are largely responsible for the damage to the bronchial tissues, thus encouraging more inflammation and precipitating bronchial hyperreactivity (Laitinen *et al*., 1985; Motijima *et al*., 1989). The present results therefore suggest that selective A_3 agonists may be useful as inhibitors of allergic inflammation.

In summary, the current results further confirm the presence of A_3 receptors on human eosinophils and shows that this receptor subtype mediates the inhibition of both degranulation and O_2^- release. Taken together with the recently reported A_3-mediated inhibition of eosinophil chemotaxis (Walker *et al*., 1997; Knight *et al*., 1997), the results suggest that selective A_3 agonists may be valuable as anti-inflammatory drugs, potentially useful in the treatment of asthma and other diseases in which activated eosinophils play a part.

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