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Effect of PDE4 inhibitors on zymosan-induced IL-8 release from human neutrophils: synergism with prostanoids and salbutamol

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1 The activation of neutrophils with particulate stimuli such as zymosan induces the generation of the C-X-C chemokine interleukin (IL)-8. There is evidence that neutrophil derived IL-8 plays an important role in human diseases such as the adult respiratory distress syndrome. In the present study, we examined the effects of cyclic AMP elevating agents on the ability of human neutrophils to generate IL-8 in response to zymosan particles.

2 The PDE4 inhibitor rolipram had limited effect on zymosan-induced IL-8 generation. In contrast, the PDE4 inhibitors RP 73401 and SB 207499 concentration-dependently suppressed IL-8 generation. The potency of these inhibitors was RP 73401 > SB 207499 > rolipram which is correlated with their rank order of potency at inhibiting the catalytic site of purified neutrophil PDE4. Pretreatment of neutrophils with the PDE3 inhibitor ORG 9935 or the PDE5 inhibitor zaprinast had no effect on IL-8 generation.

3 The prostanoids prostaglandin E_1 (PGE_1) and PGE_2 inhibited zymosan-induced IL-8 release from neutrophils in a dose-dependent manner, in response to 10^{-5}M PGE_1 and PGE_2 inhibiting IL-8 generation by 89% and 75%, respectively. Similarly, the β_2-adrenoceptor agonist salbutamol also inhibited IL-8 generation, but it was less effective than the prostanoids.

4 Significant synergism between prostanoids or salbutamol and the PDE4 inhibitors to inhibit IL-8 generation was observed. In contrast, there was no significant synergism between PGE_2 and the PDE3 inhibitor ORG 9935 or the PDE5 inhibitor zaprinast.

5 In order to evaluate the potential role of protein kinase A in mediating the inhibitory effects of cyclic AMP-elevating agents, we used the protein kinase A inhibitors, H 89 and KT 5720. Pretreatment of neutrophils with these drugs completely reversed the inhibitory effects of a combination treatment with rolipram and PGE_2 on zymosan-induced IL-8 release.

6 Microscopic examination revealed that most neutrophils contained one or more zymosan particles and that combination treatment with rolipram and PGE_2 noticeably reduced the number of ingested particles. Moreover, there was a significant reduction in the percentage of neutrophils which ingested three or more zymosan particles.

7 Thus, our results demonstrate that cyclic AMP-elevating agents modulate the ability of neutrophils to generate IL-8 in response to a particulate stimulus. However, these agents also modulate the ability of neutrophils to phagocytose zymosan particles. Whether this effect will translate into inhibition of the ability of neutrophils to deal with infectious agents needs to be investigated further.

Keywords: Neutrophils; zymosan; IL-8; PDE4 inhibitors; prostanoids; cyclic AMP/PKA pathway; phagocytosis

Introduction

Neutrophils play an essential role in local host defence against invading microorganisms. In response to chemical signals generated by the affected tissue, neutrophils migrate to the tissue where they recognize, phagocytose and destroy the foreign agent. During the phagocytic process, neutrophils release various inflammatory mediators which contribute to the local inflammatory response, including lipid mediators (eg. platelet activating factor (PAF)), reactive oxygen species, and chemokines such as interleukin (IL)-8. Under normal circumstances, the invading pathogen is destroyed, the acute inflammatory response is resolved and tissue reorganization occurs. However, persistent and/or uncontrolled activation of neutrophils may lead to tissue injury and disease.

In the adult respiratory distress syndrome (ARDS), massive recruitment of neutrophils into alveoli is an important early feature of the condition (Torre *et al*., 1993). In the lung, neutrophils secrete proteases and reactive oxygen species which cause injury to endothelial cells and lead to pulmonary oedema, an early hallmark of ARDS (Worthen & Downey, 1996). Amongst the mediators produced early in the course of the disease, the C-X-C chemokine IL-8 which is a potent neutrophil chemoattractant and activator (Baggiolini & Clark-Lewis, 1992) is of particular interest (Kunkel *et al*., 1991; Miller *et al*., 1992; Strieter & Kunkel, 1994; Chollet-Martin *et al*., 1996). Since neutrophils produce considerable amounts of IL-8 (Bazzoni *et al*., 1991; Strieter *et al*., 1992; Au *et al*., 1994), it is possible that a positive feed-back loop involving this chemokine may occur in the lung. Despite the importance and frequency of ARDS worldwide and despite a greater recent knowledge in the understanding of the disease, no therapeutic options of proven efficacy exist (Griffiths & Evans, 1996). Glucocorticosteroids may be effective in some situations but these drugs induce serious side effects, such as immunosuppression and metabolic disturbances, which diminish their efficacy

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and prevent their widespread use (Griffiths & Evans, 1996). A good therapeutic option in the treatment of ARDS should diminish neutrophil activation and neutrophil-dependent lung injury without impairing the ability of this cell to phagocytose and destroy bacteria and other invading microorganisms.

Recently, there has been much interest in the anti-inflammatory activity of phospodiesterases (PDE), a family of enzymes responsible for the metabolism of cyclic nucleotides (Beavo, 1988; Beavo *et al*., 1994). In particular, studies have focused on PDE4 isoenzymes, since these are the main isotype present in leukocytes (Nielson *et al*., 1990). Inhibitors of PDE4 induce an elevation in the intracellular levels of adenosine 3′ : 5′-cyclic monophoshate(cyclic AMP) in neutrophils and, via this mechanism, suppress several neutrophil functions including the respiratory burst (Nielson *et al*., 1990; Schudt *et al*., 1991), production of lipid mediators (Takenawa *et al*., 1986; Schudt *et al*., 1991) and degranulation (Brandt *et al*., 1992; Darius *et al*., 1994). Further, inhibitors of PDE4 have been show to suppress neutrophil-mediated injury in several animal models (Turner *et al*., 1993; Miotla *et al*., 1997). Thus, these drugs may potentially be useful in the treatment of diseases, such as ARDS, where neutrophils play an important pathophysiological role (Teixeira *et al*., 1997).

We have previously shown that human neutrophils release IL-8 when activated *in vitro* with zymosan particles (Au *et al*., 1994). Zymosan-induced IL-8 production is first detected at 8 h and is maximum 24 h after neutrophil stimulation (Au *et al*., 1994). The IL-8 is newly synthesized and its release is dependent on the integrin CD11/CD18 present on the neutrophil surface and on endogenous generation of platelet-activating factor (PAF) (Au *et al*., 1994). In the present study, we have evaluated the effects of three different PDE4 inhibitors, namely rolipram, SB 207499 and RP 73401 on IL-8 generation from human neutrophils activated with zymosan particles. Moreover, possible synergistic effects between these inhibitors and prostaglandins of the E-series or the β_2-adrenoceptor agonist salbutamol were also investigated.

Methods

Neutrophil preparation

Human neutrophils were isolated as described previously (Nourshargh *et al*., 1992). Briefly, whole blood was collected from healthy donors into acid citrate dextrose. Red blood cells were sedimented by incubation with hydroxyethylstarch (Hespan) for 1 h. Neutrophils were then separated from the mononuclear cells and the remaining red blood cells by centrifugation through a two layer discontinuous Percoll gradient (70%/81%; 1,207*g*; 30 min). Purity of the neutrophil preparation was greater than 98% with contaminating cells being predominantly eosinophils. Cells were washed three times in Ca^{2+} and Mg^{2+} free PBS before resuspension in RPMI 1640 containing 10% heated inactivated FCS.

Experimental protocol

Neutrophils (2×10^6 cells/well) were plated out in 24-well tissue culture plates (NUNC) in a final volume of 500 μl/well. Cells were pretreated with increasing concentrations of rolipram ($10^{-8}-10^{-5}$M), RP 73401 ($10^{-11}-10^{-6}$M), SB 207499 ($10^{-8}-10^{-5}$M), PGE_1 ($10^{-8}-10^{-5}$M), PGE_2 ($10^{-8}-10^{-5}$M) or salbutamol ($10^{-8}-10^{-5}$M) for 10 min at 37°C. For combination treatment, neutrophils were initially treated with increasing concentrations of rolipram, RP 73401 or SB 207499, followed by the addition of PGE_2 (10^{-7} M) or vehicle. To examine the effect of PDE3 and PDE5 inhibitors on IL-8 generation, neutrophils were pretreated with ORG 6635 (10^{-5} M) or zaprinast (10^{-5} M) alone or in combination with PGE_2 (10^{-7} M). Zymosan (10^7 particles/well) was then added to the individual wells and cells were incubated in a controlled environment (5% CO_2, 95% O_2, 37°C). Polymixin B sulphate (40 nM) was routinely added to each sample to inhibit any LPS contamination. After a 24 h incubation, cell-free culture supernatant was obtained by centrifugation at 300 *g* for 10 min. Samples (400 μl) were collected and stored at −20°C for subsequent measurement of IL-8 by radioimmunoassay (RIA).

In experiments to examine the role of the cyclic AMP/protein kinase A pathway in mediating the effects of cyclic AMP-elevating agents on IL-8 production, the protein kinase A inhibitors (H 89 and KT 5720, 10^{-8} to 10^{-5} M) were added to the neutrophils 5 min before the addition of a combination of rolipram and PGE_2 (10^{-7} M) and cells were incubated for a further 10 min before exposing the cells to zymosan. Cell-free supernatant was collected 24 h after stimulation with zymosan and assayed for IL-8 as described below.

RIA for human IL-8

Immunoreactive IL-8 concentration in samples of the 24 h cell-free supernatant was measured with a specific human IL-8 RIA, as described previously (Cromwell *et al*., 1992). Samples (100 μl) were mixed with 50 μl [^{125}I]-human IL-8 (0.5 ng) and 50 μl of goat anti-human IL-8 anti-serum (1/2000 dilution). After a 24 h incubation at room temperature, 25 μl of a second antibody, donkey anti-goat IgG (1/20 dilution), was added to each sample. After a further incubation overnight at room temperature, the competitive reaction was stopped by addition of PBS/azide (1 ml) and immediate centrifugation at 5422 *g* for 10 min. Following aspiration of the supernatant, pellets were counted in a gamma-counter. IL-8 concentration in each sample was determined by reference to a standard curve for human IL-8 established over a concentration range of 10 to 10,000 pM. Non-specific binding (NSB; 2.98 ± 0.12% of total binding; $n = 8$) was determined by incubating the labelled ligand under identical conditions but in the absence of antiserum. All samples were assayed in duplicate.

Phagocytosis assay

Neutrophils (2×10^6 cells/well) were plated out in 24-well tissue culture plates (NUNC) in a final volume of 500 μl/well. Zymosan was boiled, washed, sonicated and resuspended at 10^8 particles/well in RPMI 1640. Cells were stimulated with zymosan (10^7 particles/well) for 30 min, samples were collected and cytospin preparations (100 μl; 10^5cells; 300 *g* for 5 min) made. Slides were fixed in methanol for 5 min and stained with Hema 'Gurr' stains. In each sample, the number of neutrophils with one to three, more than three, or no phagocytosed zymosan particles was calculated by counting at least 500 cells and this was used as an index of phagocytosis. Each experiment was repeated at least three times with cells from different donors.

Statistical analysis

Data were analysed by use of a statistical software package (Instat II) and expressed as mean ± s.e.mean. Statistical analysis was performed by Dunnett's and a significant difference between groups was considered if P values were < 0.05.

Reagents

Zymosan A (from Saccharomyces cerevisae), prostaglandin (PG) E_1 and PGE_2, salbutamol sulphate,protamine sulphate, bovine serum albumin, polymixin B sulphate, sodium azide and Percoll were purchased from the Sigma Chemical Co (Poole, UK). Polyethylene glycol 6000 (PEG) and Hema 'Gurr' stains were from Merck Ltd (Poole, UK). H 89 (N-[2-(p-bromocinnamytamino)ethyl)]-5-iso-quinoline sulphonamide) and KT 5720 (8R*, 9S*, 11S*-(−)-9-hydroxy-9-n-hexyloxy- 8-methyl-2,3, 9,10 - tetrahydro - 8,11-epoxy - 1H, 8H,11H - 2,7b,11a - triazadibenzo - (a,g) - cy cloocta + + -(c,d,e)-trinden-1-one) were from Calbiochem-Novabiochem Ltd (Nottingham, UK). Hydroxyethyl starch (Hespan) was from Du Pont Pharmaceutical Ltd, Hertfordshire, UK. Foetal calf serum (FCS), calcium (Ca^{2+}) and magnesium (Mg^{2+}) free phosphate buffered saline (PBS) and RPMI 1640 culture medium containing L-glutamine and antibiotic-antimycotic (penicillin, streptomycin, and amphotericin) were from Gibco Ltd (Scotland, UK). Donkey anti-goat IgG was from Nordic Immunological Laboratories (Tilberg, The Netherlands). The following were generous gifts; human recombinant IL-8 was from Dr I. Lindley (Novartis, Austria), goat anti-human IL-8 antiserum was from Dr H. Showell (Pfizer Central Research, Connecticut, USA), rolipram, zaprinast and RP 73401 (3-cyclopentyloxy-N-[3,5-dichloro-4-pyridyl]-4-methoxybenzamide) was from Dr J. Fozard (Novartis, Switzerland), ORG 9935 (4,5-dihydro-6- (5,6-dimethoxy-benzo[b]thien - 2 - yl) - 5-methyl-1(2H) - pyridazinone) was from Dr Shahid, (Organon Laboratories, Scotland) and SB 207499 (c-4-cyano-4-(3-cyclopentyloxy - 4 - methoxyphenyl) -r-1-cyclohexanecarboxylic acid) was from Dr N. Cooper (Chiroscience, Cambridge, UK).

Results

Effect of PDE4 on IL-8 release from neutrophils activated with zymosan particles

The effects of three structurally different PDE4 inhibitors on the generation of IL-8 by human neutrophils are shown in Figure 1. Rolipram was a weak inhibitor of IL-8 generation when used alone, inhibiting IL-8 generation by 43% at 10^{-5}M (Figure 1a). In contrast, the PDE4 inhibitors, SB 207499 and RP 73401 suppressed IL-8 generation in a concentration-dependent manner, complete inhibition being observed with 10^{-5}M SB 207499 and 10^{-7}M RP 73401 (Figures 1b and c). RP 73401 was approximately 100 fold more potent than SB 207499 and 1000 fold more potent than rolipram. None of these PDE4 inhibitors had any significant effect on cell viability at the concentration used, as assessed by trypan blue dye exclusion (>90%; data not shown).

PGE_2, at a concentration that had no significant inhibitory effect alone (10^{-7}M), acted synergistically with all of the PDE4 inhibitors to suppress IL-8 generation from zymosan-stimulated neutrophils (Figure 1). The same relative potencies for the inhibition (RP 73401 > SB 207499 > rolipram) were observed in the presence or absence of PGE_2.

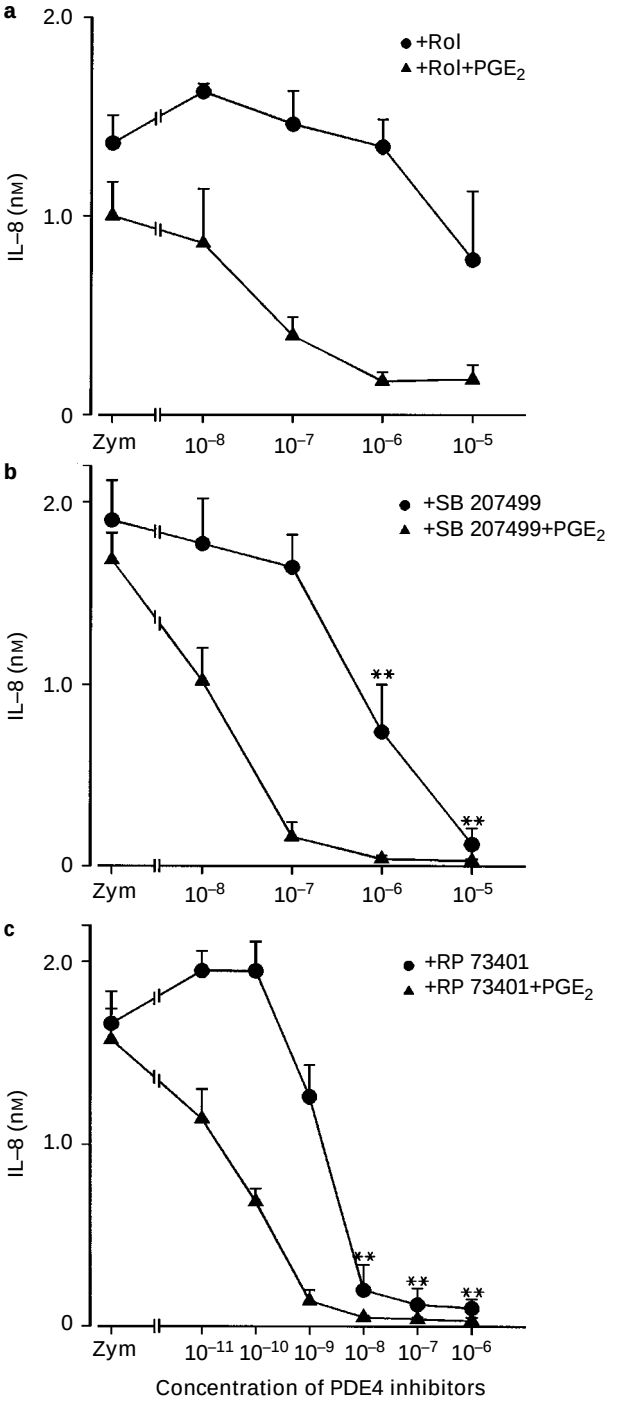

Figure 1 Effect of pretreatment with PDE4 inhibitors alone or in combination with PGE_2 on the generation of IL-8 by zymosan-activated neutrophils. Neutrophils were pretreated with increasing concentrations of (a) rolipram (10^{-8} to 10^{-5}M), (b) SB 207499 (10^{-8} to 10^{-5}M) or (c) RP 73401 (10^{-11} to 10^{-6}M) alone or in combination of PGE_2 (10^{-7}M) for 5 min. Cells were stimulated with zymosan (10^7 particles per well) for 24 h. Cell-free supernatant was collected and assayed for human IL-8. Data are expressed as mean of $n=3-6$; vertical lines show s.e.mean. Significant inhibition by the PDE4 inhibitors is indicated by $^*P<0.05$ or $^{**}P<0.01$.

Synergistic effect between prostanoids or salbutamol and PDE4 inhibitors to inhibit IL-8 generation

The effect of PGE_1 and PGE_2 on the generation of IL-8 by human neutrophils is shown in Figure 2. Both prostanoids suppressed the generation of IL-8 by zymosan-stimulated neutrophils when used at a concentration of 10^{-6}M or above (Figure 2). The maximal inhibition of IL-8 generation induced by PGE_1 and PGE_2 was 89% and 75%, respectively. The β_2-adrenoceptor agonist salbutamol was less effective than the two prostanoids, only partially inhibiting (46% at 10^{-5}M) the generation of IL-8 by neutrophils (Figure 3). A combination of rolipram (10^{-7}M) and salbutamol (10^{-8}M) which alone had no effect, abolished zymosan-induced IL-8 generation (Figure 3).

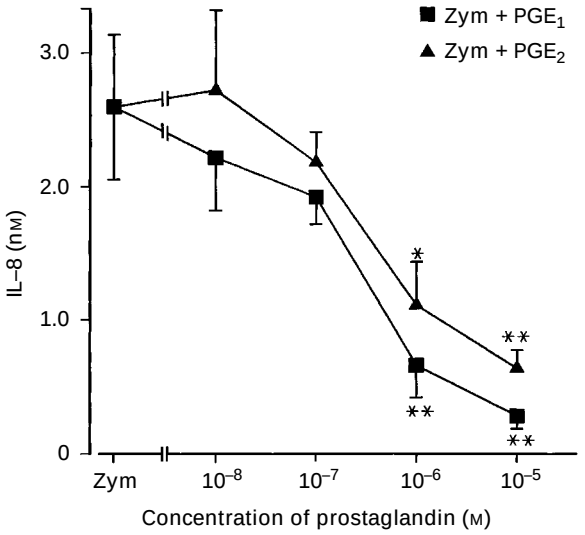

Figure 2 Effect of pretreatment with prostanoids on the generation of IL-8 by zymosan-activated neutrophils. Neutrophils were pretreated with increasing concentrations of PGE_1 or PGE_2 (10^{-8} to 10^{-5}M) for 10 min followed by activation with zymosan (10^7 particles per well). After 24 h of incubation, cell-free supernatant was collected and assayed for human IL-8. Data are expressed as mean of $n=3-6$; vertical lines show s.e.mean. Significant inhibition is indicated by $^*P<0.05$ or $^{**}P<0.01$.

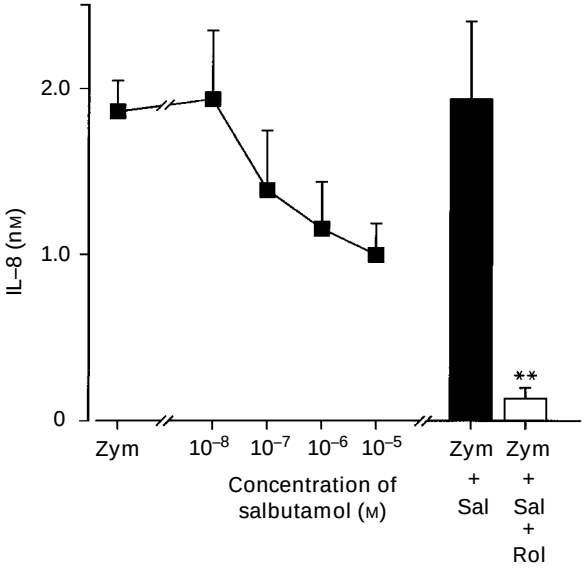

Figure 3 Effect of pretreatment with salbutamol alone or in combination with rolipram on the generation of IL-8 by zymosan-activated neutrophils. Neutrophils were pretreated with increasing concentrations of salbutamol (10^{-8} to 10^{-5}M) for 10 min. In some experiments, cells were pretreated with salbutamol (10^{-8}M) in the absence or presence of rolipram (10^{-7}M). This was followed by activation with zymosan (10^7 particles per well). After 24 h of incubation, cell-free supernatant was collected and assayed for human IL-8. Data are expressed as mean and s.e.mean of $n=4-5$. $^{**}P<0.01$, when compared to rolipram-treated cells.

The effect of the PDE3 inhibitor ORG 9935 and the PDE5 inhibitor zaprinast was also examined. Both inhibitors failed to alter significantly the zymosan-induced generation of IL-8 by neutrophils (Table 1). In contrast to the synergistic effect of the PDE4 inhibitors and prostanoids, pretreatment of neutrophils with a combination of ORG 9935 or zaprinast and PGE_2 had no significant effect on IL-8 generation (Table 1).

Because there was significant synergism between PGE_2 and rolipram at concentrations (10^{-7}M each) at which neither of these drug had any effect alone (Figures 1a and 2), this combination was used in further experiments.

Table 1 Effects of PDE3, PDE4, and PDE5 inhibitors alone or in combination with PGE_2 on IL-8 release from human neutrophils activated with zymosan particles

	IL-8 generation (nM)	
	Alone	*+ PGE_2*
Zymosan	1.71±0.14	1.63±0.07
+ ORG 9935 (PDE3)	1.43±0.04	1.20±0.19
+ Rolipram (PDE4)	1.48±0.14	0.14±0.19**
+ Zaprinast (PDE5)	1.49±0.11	1.44±0.11

Neurophils were pretreated with the PDE3 inhibitor ORG 9935 (10^{-5}M), the PDE4 inhibitor rolipram (10^{-7}M) or the PDE5 inhibitor zaprinast (10^{-5}M) for 5 min followed by PGE_2 (10^{-7}M) for another 5 min. Cells were then activated with zymosan (10^7 particles per well) and, after 24 h of incubation, cell free supernatant was collected and assayed for human IL-8. Data are expressed as mean±s.e.mean of $n=4-5$. Significant inhibition in the presence of PGE_2 is indicated by $^{**}P<0.01$.

Effect of PKA inhibitors on the regulation of zymosan-induced IL-8 release by rolipram and PGE_2

In order to confirm that the inhibitory effect of the rolipram + PGE_2 combination was mediated by a protein kinase A pathway, two inhibitors of protein kinase A were used, H 89 and KT 5720 (Irie *et al*., 1985; Drake & Issekutz, 1993). As seen in Figure 4, pretreatment of neutrophils with either H 89 or KT 5720 completely reversed the inhibitory effects of combined treatment with rolipram + PGE_2 on IL-8 generation induced by zymosan. Unstimulated cells or cells treated with H 89 or KT 5720 alone did not produce IL-8 over 24 h. Moreover, at the concentrations used, the two protein kinase A inhibitors had no effect on the viability of neutrophils (data not shown).

Effect of combined treatment with rolipram + PGE_2 on the phagocytosis of zymosan particles by neutrophils

Figure 5(a and b) shows the histological appearance of control and rolipram + PGE_2-treated neutrophils 30 min after the addition of zymosan. Whereas most control cells contained several zymosan particles, rolipram + PGE_2-treated neutrophils had noticeably fewer or no particles. In order to quantify the degree of inhibition of phagocytosis by these drugs, the percentage of neutrophils which had ingested zymosan particles was counted microscopically. The pretreatment of neutrophils with rolipram + PGE_2 inhibited the number of neutrophils undergoing phagocytosis by 53% (Figure 5c). In addition, the percentage of cells which ingested more than 3 zymosan particles was significantly less in rolipram + PGE_2-treated cells than vehicle-treated cells (Figure 5c). At the concentration used (10^{-7}M), pretreatment with rolipram or PGE_2 alone failed to alter the ability of neutrophils to phagocytose zymosan particles (5% and 8% inhibition, respectively).

Discussion

There is much evidence to suggest a role for uncontrolled neutrophil activation in the pathophysiology of various chronic and acute diseases (Brennan *et al*., 1990; Nickoloff

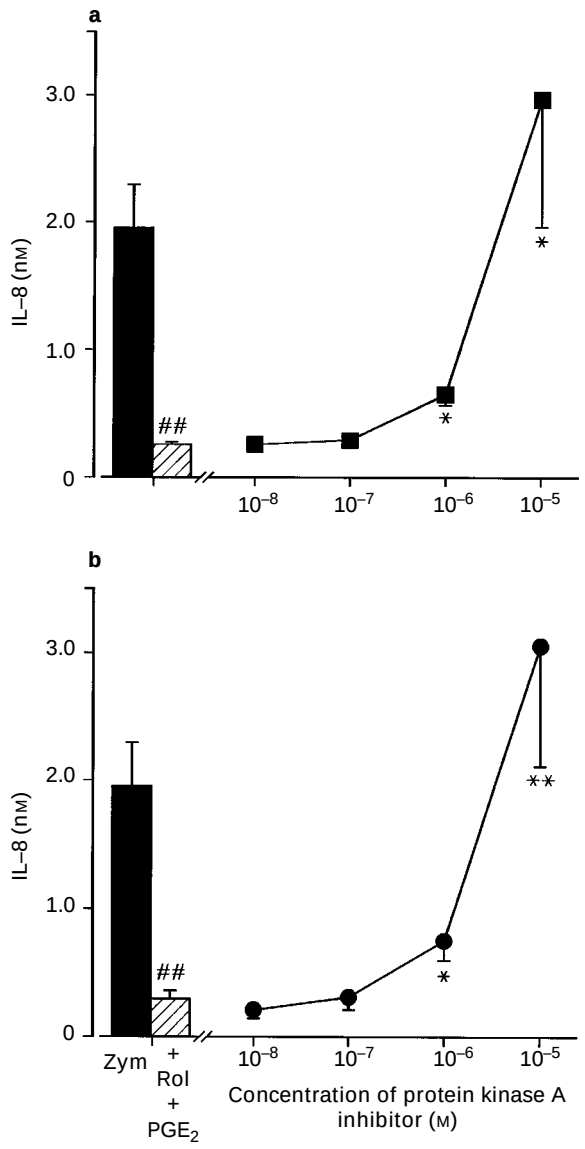

Figure 4 Effect of protein kinase A inhibitors on the suppressive effects of combined treatment with PGE_2 and rolipram on the generation of IL-8 by zymosan-activated neutrophils. Neutrophils were pretreated with increasing concentrations of (a) H 89 (10^{-8}–10^{-5}M) and (b) KT 5720 (10^{-8}–10^{-5}M) for 5 min followed by PGE_2 (10^{-7}M) and rolipram (10^{-7}M) for another 10 min. Cells were then activated with zymosan (10^7 particles per well) and, after 24 h of incubation, cell-free supernatant was collected and assayed for human IL-8. Data are expressed as mean and s.e.mean of $n=4-5$. Significant inhibition by rolipram + PGE_2 is indicated by $\#P<0.0$, $*P<0.05$, $**P<0.01$, when compared to rolipram + PGE_2-treated cells.

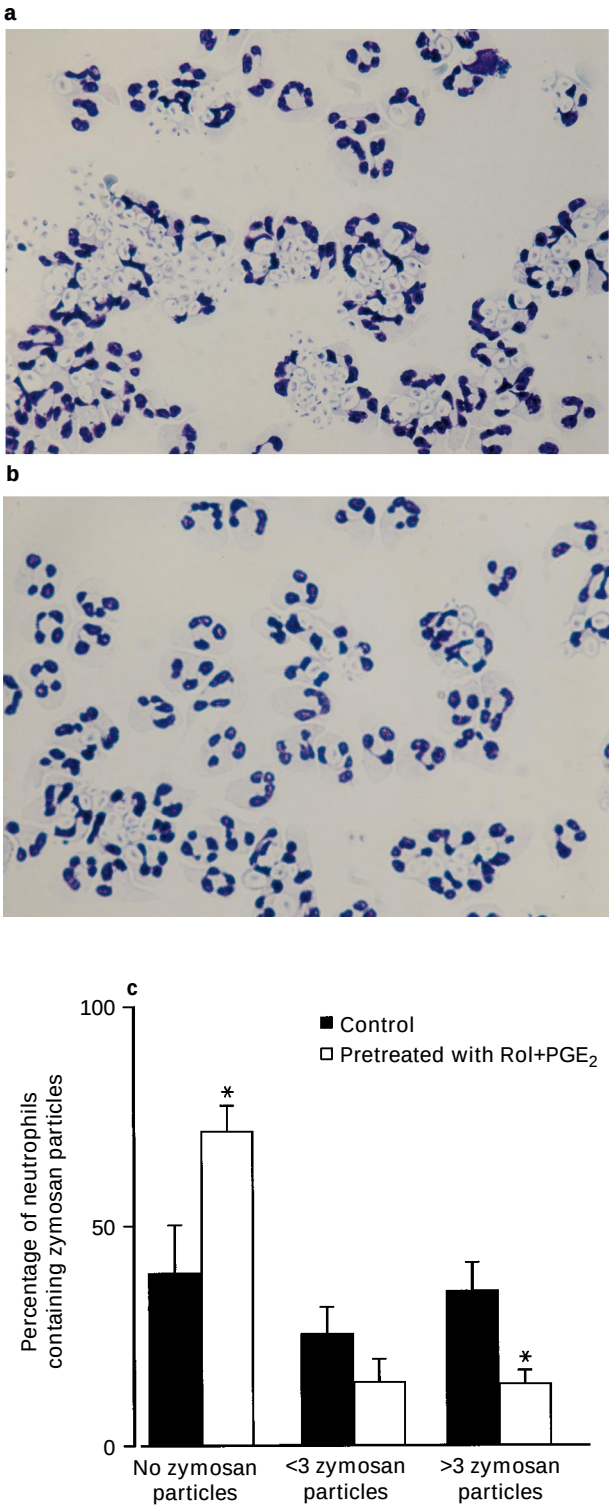

Figure 5 Effects of combined treatment with PGE_2 and rolipram on the phagocytosis of zymosan particles by neutrophils as assessed by light microscopy. Cells were pretreated with PGE_2 + rolipram and then stimulated with zymosan for 30 min. Cytospins were made and slides were fixed and stained with Hema 'Gurr' stains. (a) Most of the neutrophils treated with vehicle have several zymosan particles inside; (b) when neutrophils were treated with PGE_2 + rolipram, there was a marked decrease in the number of cells which had ingested particles and most neutrophils with particles had ingested fewer than 3 particles. In (a) and (b) bar = 30 μm. (c) Quantitative analysis of neutrophils (expressed as percentage of total neutrophils) which have ingested no particles, less than 3 and 3 or more zymosan particles. Data are presented as mean ± s.e.mean of $n=5$ donors. $*P<0.05$ when compared to control.

et al., 1991; Rampart *et al*., 1992; Torre *et al*., 1993). Among the mediators capable of activating neutrophils, the chemokine IL-8 appears to be of particular importance, not only because it is a potent neutrophil chemoattractant and activating factor but also because neutrophils themselves can produce considerable amounts of IL-8 (Bazzoni *et al*., 1991; Strieter *et al*., 1992; Au *et al*., 1994). In the present study, we have evaluated the effects of phosphodiesterase inhibitors and other cyclic AMP-elevating agents on the ability of neutrophils to release IL-8 in response to activation with zymosan particles.

Three classes of compounds known to induce an elevation of cyclic AMP in neutrophils were used – PDE4 inhibitors, prostaglandins of the E series, and a β_2-adrenoceptor agonist (Rivkin *et al*., 1975; Takenawa *et al*., 1986; Kuehl *et al*., 1987;

Schudt *et al*., 1991). PDE4 inhibitors such as rolipram increase cyclic AMP levels by inhibiting the metabolism of cyclic AMP. Prostaglandins and the β_2-adrenoceptor agonist increase cyclic AMP levels by activating surface receptors which are G_s protein coupled to the cyclic AMP-generating enzyme adenylate cyclase (Kammer, 1988; Nicholson & Shahid, 1994).

Rolipram, when used alone, had little effect on the production of IL-8 by zymosan-activated neutrophils. This is in agreement with the lack of effect of rolipram when used alone on various neutrophil functions, including the respiratory burst (Nielson *et al*., 1990; Schudt *et al*., 1991). In contrast, two newly described PDE4 inhibitors, RP 73401 and SB 207499, dose-dependently and completely inhibited IL-8 generation by neutrophils when used alone at concentrations of 10^{-7}M and 10^{-5}M, respectively. RP 73401 was approximately 100 times more potent than SB 207499. The rank order of potencies of the PDE4 inhibitors in modulating IL-8 generation when used in combination with PGE_2 is in good agreement with their rank order of potency at inhibiting the catalytic site of purified neutrophil PDE4 (Barnette *et al*., 1994; Cohan *et al*., 1996; Muller *et al*., 1996).

The significant synergism between PDE4 inhibitors and PGE_2, or rolipram and salbutamol is in good agreement with other observation of synergistic interactions between agents which activate adenylate cyclase and suppress other neutrophil functions *in vitro* (Nielson *et al*., 1990; Nicholson & Shahid, 1994). In contrast to the effect of PDE4 inhibitors, a PDE3 and PDE5 inhibitor had no inhibitory effect on zymosan-induced IL-8 production alone or in combination with PGE_2. This correlates with the lack of expression of PDE3 and PDE5 in neutrophils and suggests that PDE4 is the main isoenzyme responsible for the regulation of cyclic AMP and the generation of IL-8 in human neutrophils. Recently, Zurbonsen *et al*. (1997) showed that the antiproliferative effects of some PDE4 inhibitors on the Dami cell line was due to their cytotoxic effect rather than their effects on cyclic AMP levels. However, since we failed to observe any significant effect of PDE4 inhibitors on neutrophil viability, this could not account for the inhibitory effect of these drugs on IL-8 production from neutrophils.

We evaluated the potential role of a cyclic AMP/PKA pathway in regulating IL-8 production by using two structurally distinct PKA inhibitors, H 89 and KT 5720 (Irie *et al*., 1985; Drake & Issekutz, 1993). Preincubating neutrophils with rolipram and PGE_2 blocked the zymosan-induced IL-8 release and this inhibition was dose-dependently reversed by the PKA inhibitors. This indicates that activation of the cyclic AMP/protein kinase A cascade is an effective means of preventing the production of IL-8 by zymosan-activated neutrophils. In this respect, the 5′-flanking region of the IL-8 gene isolated from monocytes has a cyclic AMP responsive element that is potentially the site of cyclic AMP regulation of monocyte-derived IL-8 (Mukaida *et al*., 1989). However, PGE_2 does not inhibit the generation of IL-8 by neutrophils activated with LPS (Wertheim *et al*., 1993), suggesting that a direct effect on the IL-8 gene may not explain in full the inhibitory effects of cyclic AMP elevating agents observed in the present study.

It has previously been found that PGE_2, methylxanthines and cyclic AMP analogues inhibit phagocytosis-induced release of β-glucuronidase by human neutrophils (Henson, 1971; Weissmann *et al*., 1971). Inasmuch as phagocytosis of zymosan particles appears to be essential for the generation of IL-8 (Bazzoni *et al*., 1991; Au *et al*., 1994), we examined the effects of a combination treatment with rolipram and PGE_2 on the ability of neutrophils to phagocytose zymosan. A significant reduction in the percentage of neutrophils which ingested zymosan particles was observed in cells treated with rolipram and PGE_2. Moreover, there was a significant reduction in the percentage of neutrophils which ingested 3 or more zymosan particles. Thus, it appears that cyclic AMP-elevating agents differentially modulate the ability of neutrophils to produce IL-8 depending on the activating stimuli. Whereas IL-8 generation induced by soluble stimuli (eg. LPS) is not affected by cyclic AMP-elevating agents (Wertheim *et al*., 1993), IL-8 generation induced by particulate stimuli (eg. zymosan) is effectively inhibited, presumably by the ability of cyclic AMP-elevating agents to inhibit phagocytosis of the activating particles.

In summary, we have shown cyclic AMP-elevating agents to be effective modulators of IL-8 generation by zymosan-activated human neutrophils. The inhibitory effects of cyclic AMP on IL-8 release from neutrophils may provide an important physiological and a clinically relevant therapeutic mechanism for limiting the release of neutrophil-derived cytokines during certain inflammatory responses *in vivo*. Amongst the agents using in the present study, there has been much interest in the use of PDE4 inhibitors as anti-inflammatory agents in various diseases in which neutrophils are thought to play a major pathophysiological role (Teixeira *et al*., 1997). Indeed, PDE4 inhibitors are effective suppressors of neutrophil functional responses, including production of inflammatory mediators, reactive oxygen species and degranulation (Nelson *et al*., 1985; Takenawa *et al*., 1986; Schudt *et al*., 1991; Darius *et al*., 1994). Furthermore, Miotla *et al*. (1997) have recently found that the PDE4 inhibitor rolipram blocked the acute lung injury in the mouse presumably via its ability to inhibit neutrophil activation. Finally, whether the ability of PDE4 inhibitors to inhibit neutrophil phagocytosis will translate to an inhibition of the ability of neutrophils to deal with infectious microorganisms in the clinical setting needs further investigation. However, at least in one study the non-specific PDE inhibitor aminophylline has been shown to reduce the ability of neutrophils to deal with bacterial infection in an animal model (Nelson *et al*., 1985).

To conclude, we believe that the ability of PDE4 inhibitors to inhibit phagocytosis could raise concerns for the use of these compounds clinically. Experimental studies should be designed to investigate whether PDE4 inhibitors block the ability of the host to deal with infectious agents because of a suppression of phagocytosis *in vivo*.

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