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Differential effects of non-selective and selective phosphodiesterase inhibitors on human eosinophil functions

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1 The effect of non-selective (3-isobutyl-1-methylxanthine, IBMX; theophylline) and type IV- or type III/IV-selective (rolipram, RP 73401; zardaverine, tolafentrine) phosphodiesterase (PDE) inhibitors on human eosinophil functions was investigated.

2 For this purpose human eosinophils were purified from blood of healthy donors by a magnetic cell separation (MACS) technique to a purity $\geqslant 99\%$. From the stimuli investigated (complement C5a; N-formyl-methionyl-leucyl-phenylalanine, fMLP; platelet activating factor, PAF; opsonized zymosan) C5a was selected to test the influence of the above mentioned compounds on secretion of granule constituents (eosinophil cationic protein, ECP; eosinophil-derived neurotoxin, EDN) as well as on formation of reactive oxygen species measured by luminol-enhanced chemiluminescence in intact cells. For comparison, inhibition of PDE IV activity in the cytosol of disrupted cells, which contains about 75% of total PDE IV activity, was determined.

3 Both theophylline and IBMX inhibited the two cell responses with IC_{50} values which were in the range of their IC_{50} values obtained for inhibition of PDE IV activity in the cell-free system. The β_2-adrenoceptor agonist, salbutamol ($1\ \mu mol\ l^{-1}$), which by itself did not substantially influence the two cell responses, only marginally improved the potency of theophylline and IBMX in inhibiting ECP/EDN secretion. Only the IC_{50} value of IBMX for inhibition of chemiluminescence was lowered by about one order of magnitude in the presence of salbutamol.

4 In contrast, none of the selective PDE inhibitors tested substantially inhibited the two cell responses at concentrations up to $10\ \mu mol\ l^{-1}$. This was surprising because all of the compounds investigated inhibited PDE IV activity in the cell-free system with IC_{50} values which were at least 30 fold lower than the highest concentration of the compounds used with intact cells. In combination with salbutamol, however, both ECP/EDN secretion and chemiluminescence was inhibited by rolipram and zardaverine with IC_{50} values similar to the IC_{50} values for inhibition of PDE IV activity. Although RP 73401 and tolafentrine also inhibited both cell responses in the presence of salbutamol, the potency of these two compounds in inhibiting eosinophil function in intact cells was at least two orders of magnitude lower than would have been expected from the inhibition of PDE IV activity in the cell-free system.

5 These results indicate that (i) C5a-stimulated human eosinophils are sensitive to inhibition by the non-selective PDE inhibitors theophylline and IBMX, (ii) the inhibitory effect of these non-selective PDE inhibitors cannot be mimicked by selective PDE IV or PDE III/IV inhibitors although human eosinophils almost exclusively contain PDE IV; (iii) the selective PDE inhibitors need an additional cyclic AMP trigger like a β_2-adrenoceptor agonist to be effective; but (iv) under the latter conditions inhibition of cell responses in intact cells does not correspond to inhibition of PDE IV activity in the cell-free system.

6 We conclude that the non-selective PDE-inhibiting xanthines may inhibit C5a-stimulated human eosinophil responses by other action(s) in addition to PDE IV inhibition, and that inhibition of PDE IV activity in the cell-free system by the selective inhibitors may not generally represent the potency of the compounds in intact cells.

Keywords: Human eosinophils; complement C5a; eosinophil cationic protein; eosinophil-derived neurotoxin; chemiluminescence; phosphodiesterase IV; phosphodiesterase inhibitors; xanthines

Introduction

In contrast to the former assumption that the basic defect in asthma lies in an abnormal contractility of airway smooth muscle, the dogma that 'asthma is a chronic inflammatory condition of the airways' has been generally accepted in the last few years (Barnes, 1992). The well-known anti-inflammatory as well as immunosuppressive actions of corticosteroids together with the efficacy of this class of drugs in the treatment of asthma (Barnes & Pedersen, 1993) provide one major piece of evidence for the importance of underlying inflammatory processes in the pathophysiology of asthma. However, the use of corticosteroids is limited by some side effects (Barnes & Pedersen, 1993), underlining the need for new anti-inflammatory drugs.

In most of the inflammatory cells elevated adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels are found to inhibit cellular responses. One way to elevate cyclic AMP within cells is to attenuate the breakdown of cyclic AMP mediated by the action of phosphodiesterases (PDEs). PDEs comprise a family of isoenzymes which have been classified into PDEs I–VI according to the nomenclature proposed by Beavo & Reifsnyder (1990). Interestingly, the predominant PDE isoenzyme in inflammatory cells is PDE IV (Giembycz, 1992) which is the only cyclic AMP-specific PDE among the classical PDE isoenzymes. Recently, another cyclic AMP-specific PDE has been cloned (Michaeli *et al.*, 1993) as a representative of a previously unknown PDE family (PDE VII) the role of which, however, has yet to be clarified. In addition to the established presence of PDE IV in inflammatory cells, a considerable number of *in vitro* func-

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tional studies indicate that inhibition of PDE IV by selective inhibitors indeed abrogates cellular responses of inflammatory cells implicated in asthma pathogenesis (Giembycz, 1992). Among these cells the eosinophilic granulocytes are accepted as playing a pivotal effector role in the generation of airway obstruction observed in asthmatics (Gleich & Adolphson, 1986). Eosinophils are not only capable of releasing a multitude of mediators (bioactive lipids, cytokines) and reactive oxygen species, but also of secreting several cytotoxic proteins (major basic protein, MBP; eosinophil cationic protein, ECP; eosinophil-derived neurotoxin, EDN) stored in the so-called specific granules (Bruijnzeel, 1989). Elevated levels of these proteins have been demonstrated in association with *in vivo* asthma symptomatology (Gleich, 1990). While the inhibition of EDN release in human eosinophils by the non-selective PDE inhibitors IBMX (3-isobutyl-1-methylxanthine) and theophylline has been reported (Kita *et al.*, 1991), little information of the effect of selective PDE inhibitors on human eosinophil responses is available. Preliminary work has been published on the effect of the mixed-type PDE III/IV inhibitor zardaverine on the respiratory burst activity (Dent *et al.*, 1990), on the effects of the PDE IV inhibitor WAY-PDA-641 on PAF-induced superoxide generation (Maruo *et al.*, 1993) and on PAF- or N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced migration (Tanimoto *et al.*, 1993), as well as on the effect of rolipram, zardaverine and the PDE III inhibitor motapizone on interleukin-5- and PAF-induced chemotactic responses (Shute *et al.*, 1993).

In the present study the effect of the selective PDE IV inhibitors rolipram (Schudt *et al.*, 1991b) and RP 73401 (Karlsson *et al.*, 1993) and the mixed-type PDE III/IV inhibitors zardaverine (Schudt *et al.*, 1991b) and tolafentrine (Schudt *et al.*, 1993) on ECP/EDN secretion as well as on formation of reactive oxygen species in human eosinophils was investigated in comparison with the non-selective PDE inhibitors, IBMX and theophylline. Apart from these functional studies in intact cells, the inhibition of PDE IV activity by these compounds in the cytosol of disrupted cells was assessed.

Methods

Isolation of human eosinophils

Fresh human blood, 5 × 50 ml (anticoagulated with 0.3% sodium citrate) from healthy donors was centrifuged at room temperature for 15 min at 200 g. After aspiration of the supernatant (platelet-rich plasma), the residue was diluted with HBSS/HEPES (composition, mmol l^{-1}: KCl 5.4, NaCl 137, Na_2HPO_4 0.3, KH_2PO_4 0.4, $NaHCO_3$ 4.2, glucose 5, HEPES 10, pH 7.4) to a total volume of 320 ml. Forty ml each was mixed with 20 ml of a 5% dextran solution (in HBSS/HEPES) and allowed to stand for about 30 min at room temperature; 25 ml of the supernatant was layered on top of 10 ml ficoll paque and centrifuged for 10 min at 800 *g*. The sediments were resuspended in HBSS/HEPES (4°C) and washed by centrifugation for 5 min at 315 *g*. The remaining red blood cells were lysed for 15 min at 4°C in a buffer containing, mmol l^{-1}: NH_4Cl 155, $KHCO_3$ 10 and EDTA 0.1 (pH 7.4). Afterwards the cells were washed first twice in HBSS/HEPES and then once in PBS/FCS (mmol l^{-1}: KCl 2.7, NaCl 137, Na_2HPO_4 8.1, KH_2PO_4 1.5, FCS 2%, pH 7.4) by centrifugation at 4°C for 5 min at 200 *g*. For the purification of eosinophils the magnetic cell separation (MACS) system from Miltenyi Biotec (Bergisch-Gladbach, Germany) was applied as described by Hansel *et al.* (1991) with some modifications. A two-step procedure was developed which reduces the consumption of anti-CD16 microbeads to about one third. Briefly, granulocytes were resuspended in PBS/FCS at a cell concentration of 10^7 cells/17.5 µl before addition of 2.5 µl anti-CD16 microbeads. After incubation for 30 min at 4°C, 10 ml PBS/FCS (4°C) was added and the cell suspension transferred to the top of a D-separation column in the magnetic field. The flow was adjusted to about 3.5 ml min^{-1} by a 3-way tap. The non-magnetically labelled cells (eosinophils) were eluted by rinsing the column with about 130 ml PBS/FCS. After this first step, the purity of the eosinophil cell suspension was 50–90%. After washing the cells in PBS/FCS (4°C) by centrifugation for 5 min at 200 *g*, the cells were resuspended in 50 µl PBS/FCS and the same volume of anti-CD16 microbeads was added. The cell suspension was again incubated for 30 min at 4°C, then 0.9 ml PBS/FCS was added and the cell suspension transferred to the top of a B_2-separation column. Eosinophils were eluted as described above with 12 ml of PBS/FCS and washed again in PBS/FCS. After this second purification step the purity of eosinophils was ≥ 99% as determined by combined May-Grünewald/Giemsa staining, and the viability was >97% as measured by Trypan blue exclusion. The yield of eosinophils was 2.13 ± 1.16 × 10^7 cells 250 ml^{-1} blood (n = 34). We did not determine eosinophil numbers of the blood donors in whole blood; however, assuming an average number of 0.17 × 10^6 eosinophils ml^{-1} blood of healthy subjects, the recovery of eosinophils by the isolation procedure employed can be estimated to be about 50%.

Secretion of ECP and EDN

All assays were performed in duplicate at a cell concentration of 10^6 cells ml^{-1} in Dulbecco's phosphate buffered saline (PBS) (pH 7.4) supplemented with 1 mmol l^{-1} Ca^{2+}/Mg^{2+}, 5 mmol l^{-1} glucose and 0.1% (w/v) BSA. The cell suspensions (1 ml) were preincubated for 10 min at 37°C before stimulation of the assays as indicated in the legends to the figures. Thereafter, the cells were spun down by centrifugation for 30 s in an Eppendorf centrifuge. Except for the inhibition experiments (see below) the supernatants were diluted 1:5 (v/v) in PBS containing 0.006% NP 40 and 1 mmol l^{-1} EGTA and stored at −20°C. The cell pellets were resuspended in an equivalent volume of PBS containing 0.5% NP 40 and 1 mmol l^{-1} EGTA and stored at −20°C. The cell pellets were diluted 1:50 (v/v) in PBS before radioimmunoassay (RIA) analysis. For the inhibition experiments the supernatants of the incubations (0.25 ml) were frozen directly and diluted 1:5 (v/v) in PBS prior to RIA analysis. Aliquots of the samples (50 µl) were taken for ECP- and EDN-measurements by a RIA method according to the manufacturers instructions.

Chemiluminescence assay

The formation of reactive oxygen species was assessed by luminol-enhanced chemiluminescence essentially as described (Schudt *et al.*, 1991a). Briefly, aliquots (0.5 ml) of the cell suspension (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C in a buffer consisting of mmol l^{-1}, NaCl 140, KCl 5, HEPES 10, $CaCl_2/MgCl_2$ 1, glucose 1 mg ml^{-1}, BSA 0.05% (w/v) luminol 10 µmol l^{-1} and microperoxidase 4 µmol l^{-1}. The assays were then transferred into a 'Multi-Bioluminat' LB 9505C from Berthold (Wildbad, Germany) before stimulation of the assays as indicated in the legends to the figures. Chemiluminescence was continuously recorded for the indicated times and the AUC's (area under the curve) calculated.

In order to assess unspecific quenching of chemiluminescence, formation of O_2^- was triggered in the absence of cells (but otherwise identical conditions) by xanthine oxidase (25 mu) in the presence of hypoxanthine (0.3 µmol l^{-1}). The chemiluminescence signal obtained under these conditions was of comparable magnitude.

PDE activity

For measuring of PDE activity under cell-free conditions, human eosinophils (10^7 cells ml^{-1}) were suspended in PBS

(pH 8.2) containing HEPES 10 mmol l^{-1} and 1 mmol l^{-1} EGTA, Mg^{2+} and β-mercaptoethanol before sonication of the cells for 1–1.5 min at 4°C using a Branson sonifier (microtip limit 1, duty cycle 10). Sonicates were centrifuged for 1 h at 100 000 *g*. Cytosolic and pellet fractions (resuspended in a corresponding volume of homogenization buffer) were stored at −20°C until measuring PDE activity.

PDE IV activity was determined as described by Thompson & Appleman (1979) with some modifications (Bauer & Schwabe, 1980). The assay mixture contained Tris-HCl 40 mmol l^{-1} (pH 7.4), $MgCl_2$ 5 mmol l^{-1}, cyclic AMP 0.5 μmol l^{-1} including [^{3}H]-cyclic AMP (about 50 000 c.p.m./assay), the indicated concentration of the inhibitor and an aliquot of eosinophil cytosol (15–25 μl) at a final assay volume of 200 μl. The assays were incubated for 30 min at 37°C and then stopped by the addition of 50 μl 0.2 N HCl. The assays were left on ice for about 10 min, incubated with 25 μg 5'-nucleotidase (*Crotalus atrox* snake venom) for 10 min at 37°C, and then loaded on QAE Sephadex A-25 columns (Econo colums, Bio-Rad, 1 ml bed volume). The columns were eluted with 2 ml ammonium formiate (pH 6.0) 30 mmol l^{-1} and the eluate was counted for radioactivity. Results were corrected for blank values (measured in the presence of denatured protein) which were below 2% of total radioactivity. The amount of cyclic AMP hydrolyzed did not exceed 20% of the original substrate concentration.

Chemicals and solutions

BSA (bovine serum albumin), complement C5a, dextran (mol.wt. 515,000), DMSO (dimethyl sulphoxide), EGTA (ethylene glycol-*bis*-[β-aminoethyl ether]*N,N,N',N'*-tetraacetic acid), fMLP (N-formyl-methionyl-leucyl-phenylalanine), glucose, hypoxanthine, IBMX, microperoxidase, nonidet P40 (NP 40), PAF (platelet activating factor), salbutamol, theophylline, xanthine oxidase and zymosan A were purchased from Sigma Chemie (Deisenhofen, Germany). HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) was obtained from Serva (Heidelberg, Germany), luminol from Boehringer (Mannheim, Germany), ficoll paque from Pharmacia LKB (Freiburg, Germany) and FCS (foetal calf serum) from Gibco (Paisley, Scotland). ^{125}I radioimmunoassays (RIAs) for ECP and EDN were purchased from Kabi Pharmacia (Freiburg, Germany). All other chemicals and solvents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Rolipram was a generous gift from Schering AG (Berlin, Germany), RP 73401 as well as zardaverine and tolafentrine have been synthesized at the chemistry facilities of Byk Gulden (Konstanz, Germany).

Opsonized zymosan was prepared by incubating zymosan A for 30 min at 37°C in human serum. The opsonized zymosan was washed three times in cold saline (0.9% NaCl in water, w/v) and stored at a concentration of 100 mg ml^{-1} at −20°C until use. Before opsonization, zymosan A had been boiled for 1.5–2 h in saline at a concentration of about 10 mg ml^{-1} and washed four times in boiling saline. A stock solution from fMLP (10 mmol l^{-1}) was prepared in DMSO and diluted 1:1000 (v/v) in the incubation buffer (see below). C5a was dissolved in water (100 μmol l^{-1}) and diluted 1:10 in buffer. A stock solution from PAF (1 mol l^{-1}) was prepared in ethanol and diluted 1:100 (v/v) in buffer. The solutions of all four stimuli were further diluted 1:100 (v/v) in the assays resulting in a final concentration of 100 nmol l^{-1} for fMLP, C5a and PAF and 1 mg ml^{-1} for opsonized zymosan, respectively. A stock solution from salbutamol (1 mmol l^{-1}) was prepared in water. Stock solutions from theophylline (25 mmol l^{-1}), IBMX (25 mmol l^{-1}) and zardaverine (10 mmol l^{-1}) were prepared in NaOH and from tolafentrine (10 mmol l^{-1}) in HCl so that the final assay concentrations of NaOH and HCl, respectively, were below 0.001 N at the highest inhibitor concentration tested. Stock solutions (10 mmol l^{-1}) of rolipram and RP 73401 were prepared in ethanol and DMSO, respectively, so that the final solvent concentration in the assays was below 0.01% at the highest inhibitor concentration tested. Appropriate dilutions of the inhibitors in water were further diluted 1:25 (v/v) in the assays to achieve the final concentrations indicated.

Statistics

Results are given as mean ± s.d. (standard deviation) from the number (*n*) of independent experiments (using cells from individual subjects) indicated. IC_{50} values were calculated from concentration-inhibition-curves by non-linear regression analysis using GraphPad InPlot (GraphPad Software Inc., Philadelphia, U.S.A.). Statistical significance (*P*) was determined by the paired *t* test (InStat, GraphPad Software Inc.).

Results

Secretion of ECP and EDN

In initial experiments the ability of fMLP, opsonized zymosan, C5a and PAF to trigger eosinophil granule release was compared. In a recent paper from Bach *et al.* (1991), possible pitfalls in the measurement of the secretion of granule proteins by human eosinophils have been described. A major problem seems to be the low recovery of granule proteins in the supernatant of stimulated cells compared to the loss of these proteins in the cell pellets. In order to address this problem under our experimental conditions, we measured EDN and ECP both in the supernatant and in the cell pellet of stimulated versus unstimulated cells (blank) after incubation of the cell suspensions for 30 min at 37°C.

We detected comparable amounts of EDN (2871 ± 833 ng/10^6 cells; $n = 6$) and ECP (2706 ± 1111 ng/10^6 cells; $n = 6$) in the cell pellet of unstimulated cells. These values are in agreement with those reported by others (Abu-Ghazaleh *et al.*, 1992). Under these conditions, the percentage of EDN and ECP measured in the supernatant of the cells was 3.7% and 2.9%, respectively. Figure 1a shows that all four stimuli tested lead to a comparable (2.5–3 fold compared to unstimulated cells) increase of both EDN- and ECP-levels in the cell supernatants. In general, EDN and ECP were released in parallel in agreement with the colocalization of these two granule proteins in the matrix of specific granules (Gleich & Adolphson, 1986). However, in the case of opsonized zymosan almost no ECP was detected in the supernatant, although a substantial portion of EDN was measured. Quantification of the loss of granule proteins in the cell pellets (expressed by the index: amount of the granule proteins in unstimulated divided by the amount in stimulated cells) revealed a similar pattern to that in the supernatants (Figure 1b). For opsonized zymosan a comparable loss of EDN and ECP was found in the cell pellet. The fact that no ECP could be detected in the cell supernatant with this stimulus remains unexplained.

Based on the amounts of the granule proteins lost in the cell pellets in stimulated versus unstimulated cells, the recovery of the proteins in the supernatants was calculated. For EDN the recovery was largely independent of the stimulus used and was found to be about 75% on average (data not shown). In contrast, for ECP the recovery was only about 20–25% on average (data not shown) with the exception of opsonized zymosan for which no ECP could be detected in the cell supernatant as already mentioned before. Despite the low recovery for ECP it is obvious from the data shown in Figure 1 that the stimulation pattern of granule proteins reflected either as increase of EDN and ECP in the cell supernatant (Figure 1a) or as decrease of the two proteins in the cell pellet (Figure 1b) is almost identical. These results suggest that the relative amounts of both proteins

measured in the supernatant of the cells are representative for the activation status of the cells concerning the secretion of EDN and ECP. Therefore, in further experiments assessing the influence of the compounds on EDN- and ECP-secretion, the supernatants of the cells were analysed.

For this purpose C5a was selected as a stimulus which showed the most reproducible and efficient response with respect to granule secretion (as shown above) and the chemiluminescence measurements (see below). As a prerequisite, the kinetics of EDN- and ECP-release were examined. Figure 2 demonstrates that the secretion of both proteins occurs very rapidly and plateaus at about 15 min Therefore, a stimulation time of 10 min was selected for the inhibition experiments.

Under these conditions both theophylline and IBMX concentration-dependently inhibited EDN- and ECP-release almost in parallel with IC_{50} values of 347/589 μmol l^{-1} and 50/51 μmol l^{-1}, respectively (Figure 3). The β_2-adrenoceptor agonist, salbutamol, did not show a significant concentration-dependent effect in the concentration-range tested (Figure 3). In contrast, none of the selective PDE IV (rolipram, RP 73401) or PDE III/IV (zardaverine, tolafentrine) inhibitors examined, substantially inhibited EDN- and ECP-release at concentrations up to 10 μmol l^{-1} (Figure 4). In combination with salbutamol (1 μmol l^{-1}), however, secretion of both proteins was inhibited by all four compounds tested in an obviously synergistic manner (Figure 4).

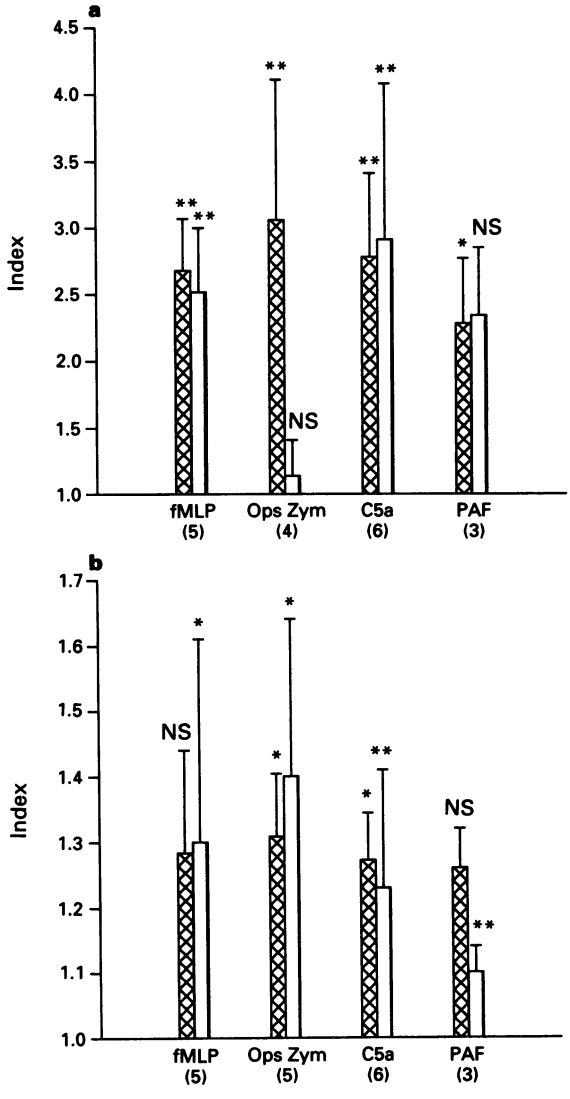

Figure 1 Stimulus-dependent secretion of eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP) by human eosinophils. Aliquots (1 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C before stimulation with fMLP, C5a, PAF (100 nmol l^{-1}, each) or opsonized zymosan (1 mg ml^{-1}). Non-stimulated cells served as blank. After 30 min the incubations were stopped by centrifugation, the supernatants were removed and the cell pellets solubilized as described in Methods. Aliquots of appropriate dilutions of the supernatants (a) and pellets (b) were assayed for EDN (crosshatched columns) and ECP (open columns) by RIA. Amounts of EDN and ECP detected in the presence of the stimuli are expressed relative to the values measured in unstimulated cells by means of an index. In the supernatant the index is defined as the ratio of the amount of EDN or ECP in stimulated cells divided by that in unstimulated cells, in the pellet *vice versa*. Data are given as mean ± s.d. from the number of independent experiments given in parentheses. Statistical significance (**$P<0.01$; *$P<0.05$; NS, not significant) using the paired t test was calculated comparing the amounts of ECP and EDN measured in stimulated versus unstimulated cells.

Chemiluminescence measurements

The formation of reactive oxygen species in human eosinophils was assessed by luminol-enhanced chemiluminescence. In analogy to the secretion experiments, the ability of fMLP, PAF, C5a and opsonized zymosan to stimulate a chemiluminescence response was investigated in initial experiments. In contrast to secretion, the chemiluminescence response differed markedly between the four stimuli which is demonstrated by a representative experiment shown in Figure 5. Whereas the soluble agonists fMLP, PAF and C5a triggered a rapid chemiluminescence signal within seconds, which declines to baseline levels in about 1–3 min, opsonized zymosan stimulated chemiluminescence with a slow onset but a long duration up to 30 min. A quantitative evaluation of all experiments performed is shown in Table 1 which summarizes the AUC's for the corresponding time-periods of the stimuli in which the most pronounced chemiluminescence signal was detected. From these data it is evident that the C5a-stimulated chemiluminescence response was most reproducible and showed the highest relative efficacy compared to unstimulated cells (blank). Therefore, inhibition experiments were

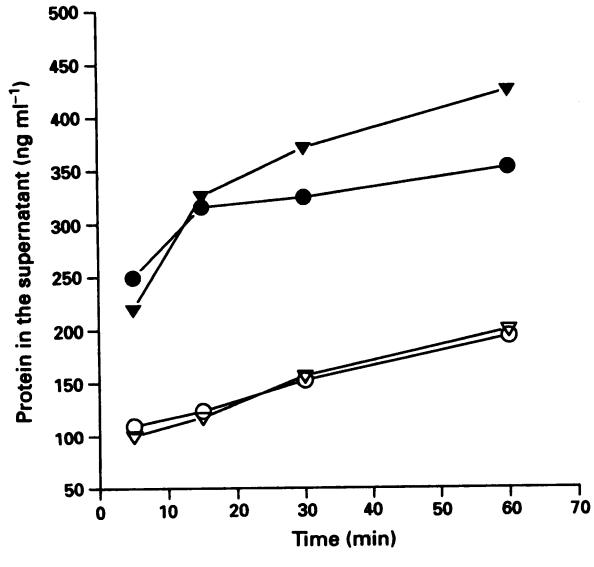

Figure 2 Kinetics of C5a-stimulated release of eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) into the supernatant of human eosinophils. Aliquots (1 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C before stimulation without (open symbols) or with 100 nmol l^{-1} C5a (solid symbols). At the indicated times the incubations were centrifuged and the amount of ECP (○,●) and EDN (△,▲) in the supernatant of the assays determined by RIA. Results are given as mean from two independent experiments.

performed with C5a using a stimulation time of 1 min. Similar to the secretion experiments both theophylline and IBMX concentration-dependently inhibited chemiluminescence with IC_{50} values of 525 μmol l^{-1} and 417 μmol l^{-1}, respectively (Figure 6). It was evident, however, that the potency of IBMX in inhibiting chemiluminescence was about one order of magnitude lower than for inhibition of secretion. Salbutamol also inhibited C5a-stimulated chemiluminescence in human eosinophils to some degree; however, almost the same inhibitory effect of salbutamol on chemiluminescence was obtained in a cell-free system consisting of hypoxanthine/xanthine oxidase (XOD) (Figure 6) which is known to produce a chemiluminescence signal by the production of superoxide anion radical (O_2^-). These results suggest that most, if not all, of the inhibitory effect of salbutamol on chemiluminescence in eosinophils is due to an unspecific quenching of O_2^- rather than to an inhibition of the production of reactive oxygen species themselves. A concentration of 1 μmol l^{-1} salbutamol had only a modest effect on chemiluminescence, therefore this concentration was used to study the combined effect of salbutamol and theophylline or IBMX, respectively, on C5a-stimulated chemiluminescence. While the effect of salbutamol on the inhibition of chemiluminescence by theophylline was simply additive (IC_{50} of 331 μmol l^{-1} versus 525 μmol l^{-1}), the IC_{50} value of IBMX for chemiluminescence inhibition was improved by more than one order of magnitude (31 μmol l^{-1} versus 417 μmol l^{-1}) in the presence of salbutamol (Figure 6).

Similar to the secretion experiments, none of the selective PDE inhibitors tested had a remarkable effect on C5a-

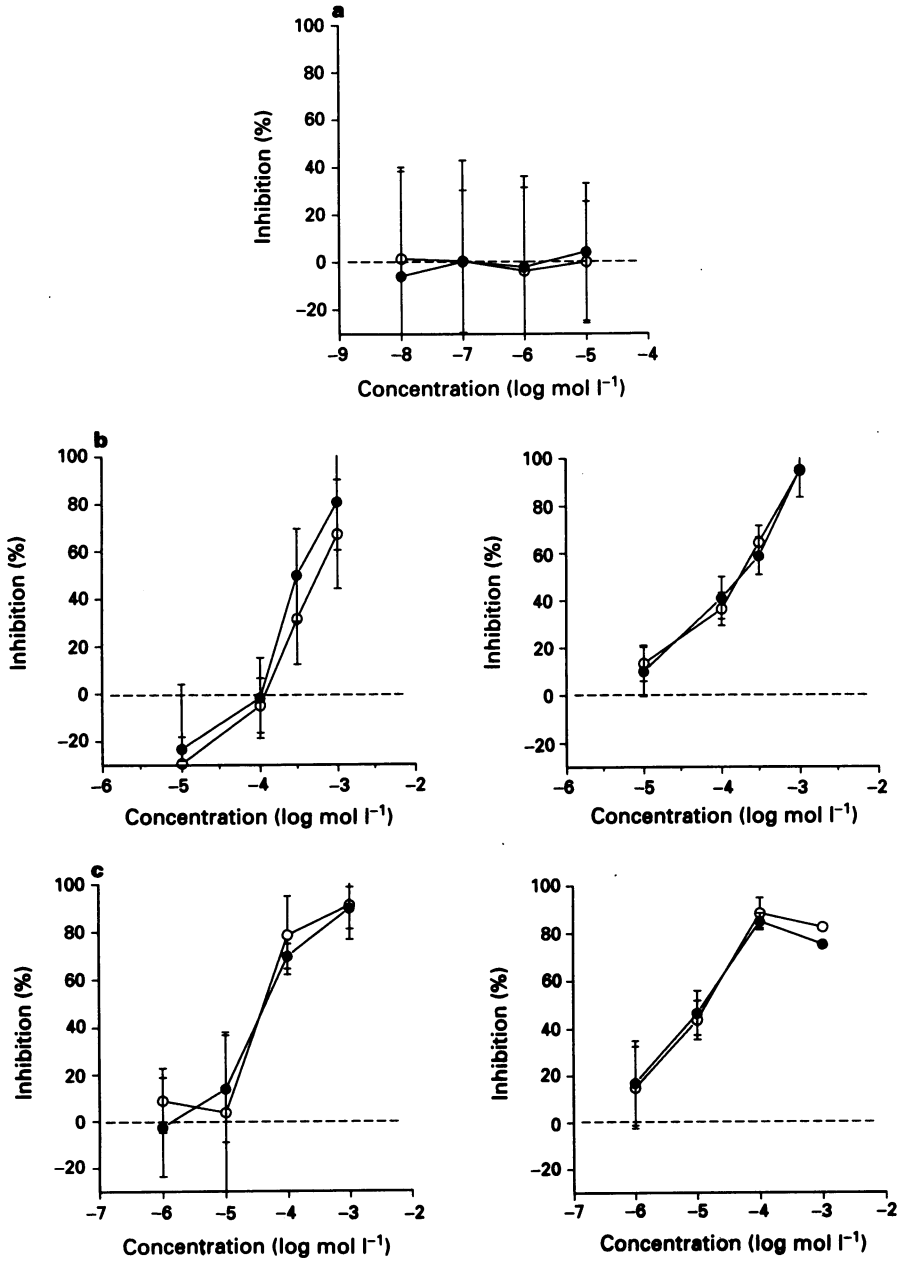

Figure 3 Influence of salbutamol (a), and theophylline (b) or 3-isobutyl-1-methylxanthine (c) (either alone left panel, or in combination with 1 μmol l^{-1} salbutamol right panel) on C5a-stimulated secretion of eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) into the supernatant of human eosinophils. Aliquots (0.25 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated either in the absence or presence of the indicated concentrations of the compounds for 10 min at 37°C. After stimulation of the assays with C5a (100 nmol l^{-1}) for 10 min, the incubations were stopped by centrifugation. Aliquots of the supernatants were taken for measurement of ECP (○) and EDN (●) by RIA. Drug effect (% inhibition) was calculated based on the amount of ECP and EDN, respectively, measured in the absence of any compound (control). Results are given as mean ± s.d. from 3–5 independent experiments.

stimulated chemiluminescence (Figure 7). Again, potency was markedly improved on combination with 1 μmol l^{-1} salbutamol which facilitates a synergistic inhibition of chemiluminescence in the presence of all four selective PDE inhibitors tested (Figure 7).

Inhibition of PDE IV activity

In initial experiments the PDE isoenzyme activity pattern as well as the subcellular localization of PDE isoenzymes was investigated. For this purpose, cyclic nucleotide hydrolyzing activity was measured with either cyclic GMP or cyclic AMP at a concentration of 0.5 μmol l^{-1}. Compared to cyclic AMP-PDE activity (235 ± 26 pmol × min^{-1} × 10^{-8} cell equivalents; $n = 10$) only trace amounts (<3%) of cyclic GMP-PDE activity (6.5 ± 3.2 pmol × min^{-1} × 10^{-8} cell equivalents) were found, indicating the absence of both PDE I and V in human eosinophils. More than 95% of total cyclic AMP-PDE activity was blocked by rolipram (10 μmol l^{-1}). The residual cyclic AMP-PDE activity was neither affected by the PDE III-selective inhibitor motapizone (1 μmol l^{-1}) nor cyclic GMP (5 μmol l^{-1}) or Ca^{2+}/calmodulin which are the

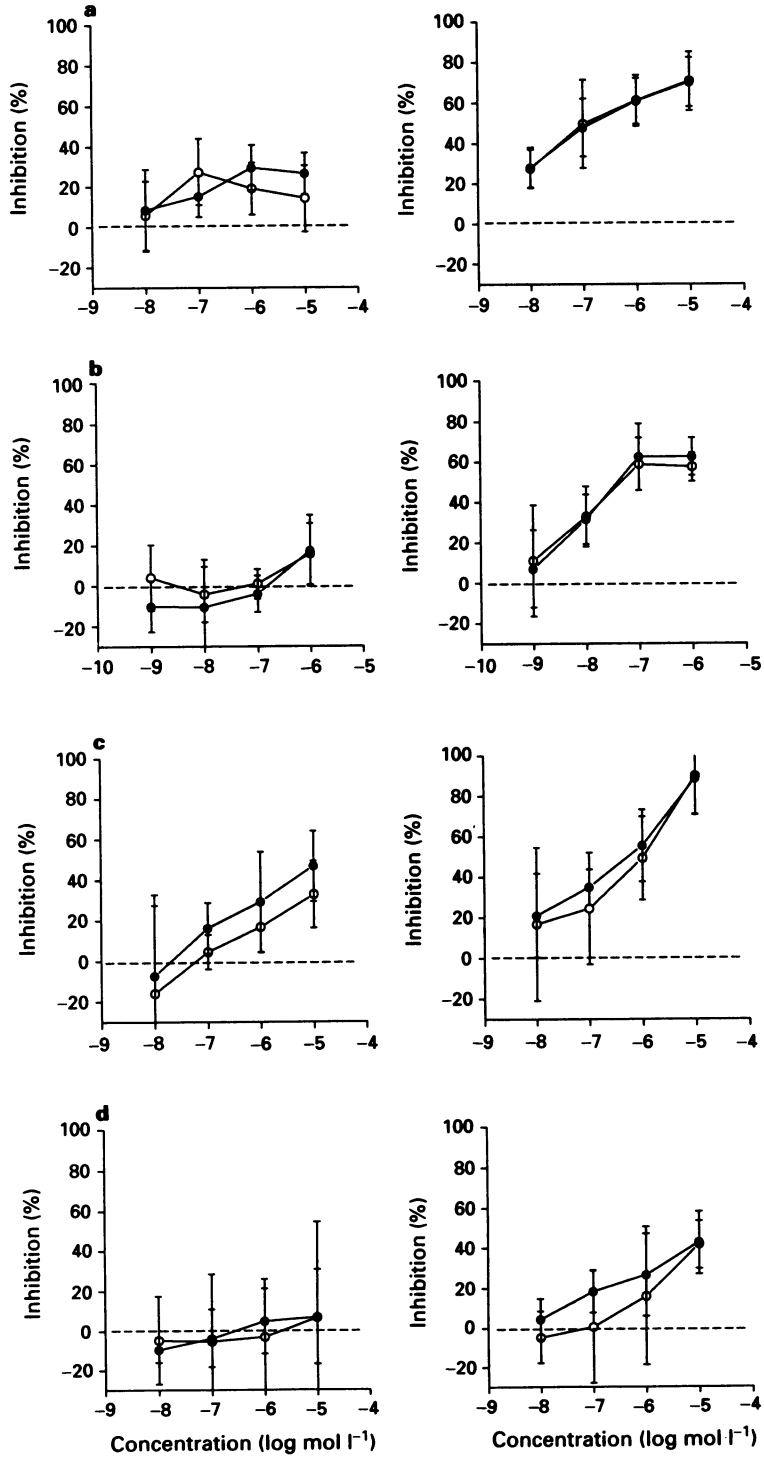

Figure 4 Influence of rolipram (a), RP 73401 (b), zardaverine (c) and tolafentrine (d) (either alone left panel, or in combination with 1 μmol l^{-1} salbutamol right panel) on C5a-stimulated secretion of eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) into the supernatant of human eosinophils. For assay conditions see legend to Figure 3. Symbols refer to ECP (○) and EDN (●), respectively. Results are given as mean ± s.d. from 3–6 independent experiments.

Table 1 Quantitative analysis of the chemiluminescence response of human eosinophils stimulated by various agonists

	Blank (AUC, c.p.m. × 10^7)	*Stimulated* (AUC, c.p.m. × 10^7)	n	*AUC Stimulated/Blank* (fold)
fMLP (0–3 min)	2.6 ± 2.6	19.4 ± 13.5	8	7.5[a]
C5a (0–1 min)	0.8 ± 1.0	14.3 ± 2.9	9	17.9[b]
PAF (0–1 min)	0.5 ± 0.4	3.1 ± 3.5	8	6.2[c]
Ops Zym (0–30 min)	46.6 ± 25.7	191.6 ± 64.2	8	4.1[d]

For assay conditions see legend to Figure 5. Data shown correspond to the AUC's (c.p.m. × 10^7) calculated for the chemiluminescence signal at time-periods typical for each agonist: fMLP 0–3 min, C5a and PAF 0–1 min, and opsonized zymosan (Ops Zym) 0–30 min. These time-periods are derived from kinetic experiments (see Figure 5), and cover the times at which a stimulus-dependent chemiluminescence signal beyond the signal of unstimulated cells (blank) was measured. Results are given as mean ± s.d. from the number of experiments indicated. Statistical significance (P) using the paired t test was calculated comparing the AUC's of stimulated versus unstimulated cells. For abbreviations, see text.
[a]$P<0.01$; [b]$P<0.0001$; [c]$P<0.05$; [d]$P=0.0001$.

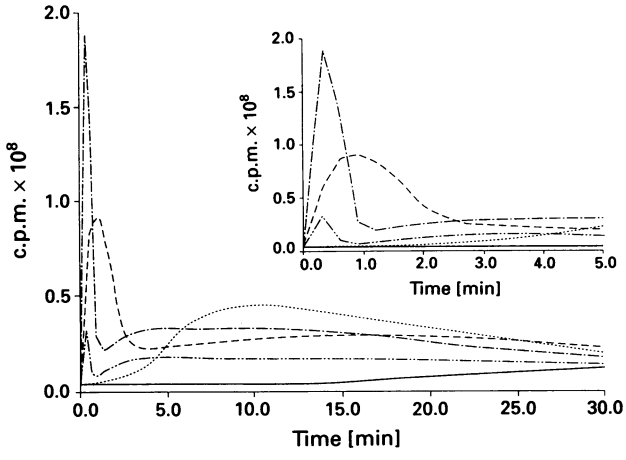

Figure 5 Stimulus-dependent chemiluminescence response of human eosinophils. Aliquots (0.5 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated for 10 min at 37°C before stimulation with 100 nmol l^{-1} fMLP (------), 100 nmol l^{-1} C5a (-·-·-), 100 nmol l^{-1} PAF (-··-··-) or 1 mg ml^{-1} opsonized zymosan (·····). Non-stimulated cells served as blank (——). Luminol-enhanced chemiluminescence was continuously recorded. The figure shows the traces of a representative experiment.

activators of PDE I and PDE II, respectively. These results indicate that PDE IV is the predominant PDE isoenzyme in human eosinophils. In addition, about 75% of PDE IV activity is located in the cytosolic fraction.

This fraction, therefore, was taken to investigate the effect of the PDE inhibitors mentioned above on PDE IV activity of human eosinophils under cell-free conditions. For all six compounds monophasic inhibition curves were obtained, the calculated IC_{50} values of which are summarized in Table 2.

A comparison of the IC_{50} values for inhibition of PDE IV activity, secretion of ECP/EDN and chemiluminescence, respectively, reveals (Table 2) that theophylline inhibited all three parameters with about the same potency. On the whole this is also true for IBMX except for the inhibition of chemiluminescence, where IBMX required the presence of salbutamol to achieve an IC_{50} value similar to that obtained for inhibition of PDE IV activity in the cell-free system. In contrast, none of the selective PDE inhibitors substantially affected intact cell responses on their own, despite the fact that these compounds were much more potent inhibitors of PDE activity in the cell-free system compared to theophylline and IBMX. Interestingly, the selective PDE inhibitors were active in the presence of salbutamol in a synergistic manner. Under these conditions, zardaverine and rolipram inhibited both ECP/EDN secretion and chemiluminescence with IC_{50} values comparable to those obtained for inhibition of PDE IV activity, while RP 73401 and tolafentrine in intact cells were one to two or at least two orders of magnitude, respectively, less potent than would have been expected from the inhibition studies of PDE activity in the cell-free system.

Discussion

The present study addressed the question whether eosinophil responses like granule secretion or formation of reactive oxygen species, which are related to the inflammatory role of these cells in asthma pathogenesis, are inhibited by PDE inhibitors *in vitro*. This class of compounds has been postulated to be promising in asthma therapy and in particular PDE IV-selective inhibitors have attracted attention because of their general potential to suppress inflammatory cell functions (Giembycz, 1992).

Although the eosinophil is accepted to play a pivotal role as effector cell in the pathogenesis of asthma, very limited information on the effect of PDE inhibitors on human eosinophil functions is available. Kita *et al.* (1991) reported the inhibition of sIgA- or IgG-stimulated EDN secretion in human eosinophils by IBMX and theophylline. The IC_{50} values for IBMX (9 and 30 μmol l^{-1}) and theophylline (90 and 500 μmol l^{-1}) reported in that study are comparable to those we have found for both compounds in the present study for C5a-stimulated ECP/EDN secretion (Table 2). In addition, we have shown that both inhibitors are effective in inhibiting formation of reactive oxygen species as measured by luminol-enhanced chemiluminescence. From Kita's studies it is evident that stimulus-dependent differences in the potency of the compounds in inhibiting human eosinophil secretion may occur. Based on initial experiments comparing different physiological stimuli, we have selected complement C5a for the inhibition experiments because under our experimental conditions the cells responded to C5a most effectively and most reproducibly. At least in the guinea-pig the importance of complement activation for antigen-induced bronchoconstriction has been considered (Regal *et al.*, 1993a,b).

We have found that human eosinophils contain PDE IV as the predominant PDE isoenzyme. Under our experimental conditions most of the PDE IV activity (75%) was recovered from the cytosol. According to our experimental protocol we used freshly isolated cells for the preparation of subcellular fractions. We have made the observation, however, that freezing (storage at −20°C) of the cells before sonication or

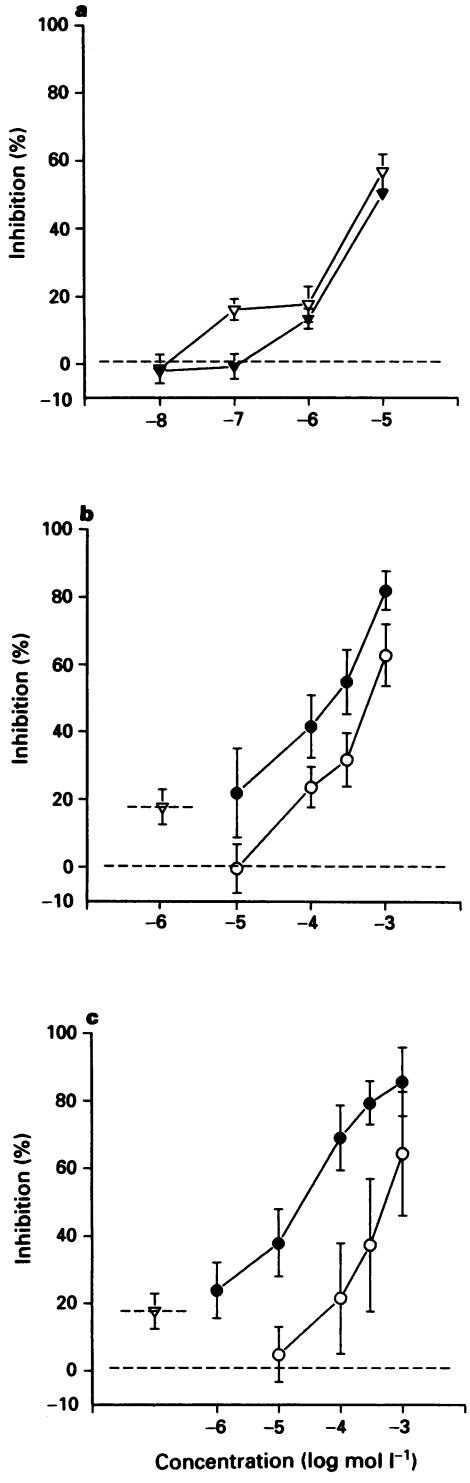

Figure 6 Influence of salbutamol (a) and theophylline (b) or 3-isobutyl-1-methylxanthine (c) (either alone or in combination with 1 μmol l^{-1} salbutamol) on C5a-stimulated chemiluminescence response of human eosinophils. Aliquots (0.5 ml) of human eosinophils (10^6 cells ml^{-1}) were preincubated either in the presence or absence of the indicated concentrations of the compounds for 10 min at 37°C. Then the assays were stimulated with C5a (100 nmol l^{-1}) for 1 min. Chemiluminescence was continuously recorded and the AUC's calculated. Drug effect (% inhibition) was calculated based on the chemiluminescence signal obtained in the absence of any compound (control). Symbols refer to the effect of PDE inhibitors alone (○) or in combination with 1 μmol l^{-1} salbutamol (●), and the effect of salbutamol itself (△), respectively. Unspecific quenching of chemiluminescence by salbutamol (a, ▲) was assessed by triggering superoxide anion formation in a cell-free system using hypoxanthine (0.3 μmol l^{-1}) and xanthine-oxidase (25 mu) under otherwise identical conditions. Results are given as mean ± s.d. from 3–5 independent experiments.

enhancement of the energy output during sonication renders total PDE IV activity to be membrane-bound (data not shown). These results indicate that the handling of the cells prior to and during disruption is a critical determinant of PDE IV localization at least in human eosinophils. Differences in the experimental procedure for the preparation of subcellular fractions may therefore account for the membrane localization of PDE IV reported by others in human (Giembycz & Barnes, 1993) as well as guinea-pig (Souness *et al.*, 1991; Dent *et al.*, 1991) eosinophils. In addition, the localization of PDE IV in eosinophils may be species-dependent.

Based on the PDE isoenzyme pattern, one would have expected that the inhibition of human eosinophil functions by the non-selective PDE inhibitors IBMX and theophylline would be mimicked by selective PDE IV inhibitors, provided that the action of IBMX and theophylline is a consequence of PDE inhibition. Surprisingly, none of the selective PDE inhibitors tested substantially inhibited ECP/EDN secretion or the chemiluminescence response, although all of these compounds were much more potent PDE IV inhibitors than theophylline or even IBMX in the cell-free system. These results are in contrast to guinea-pig peritoneal eosinophils, the O_2^--formation of which has been demonstrated to be sensitive to inhibition by selective PDE IV inhibitors both in resting cells (Souness *et al.*, 1991) and in opsonized zymosan-stimulated cells (Dent *et al.*, 1991). Whether this difference represents species-specific effects or is due to differences in the experimental conditions remains unexplained at the moment.

One explanation for the failure of the selective PDE inhibitors to inhibit human eosinophil responses could be the insufficient basal cyclic AMP concentration in these cells. Due to the requirement of large cell numbers we were not able to perform cyclic AMP measurements: however, we tried to assess the influence of an additional cyclic AMP trigger indirectly by testing the influence of a β_2-adrenoceptor agonist (salbutamol). We are not aware of any literature directly proving the existence of β_2-adrenoceptors on human peripheral eosinophils purified from healthy blood donors. However, the enhancement of IBMX-mediated cyclic AMP levels by β_2-adrenoceptor agonists like salbutamol and isoprenaline and its reversal by the β-adrenoceptor antagonist propanolol as reported in the study of Kita *et al.* (1991) provides circumstantial evidence for the presence of such receptors on human eosinophils. In addition, β-adrenoceptors of the β_2-subtype that are coupled to adenylate cyclase have been characterized in eosinophils from patients with eosinophilia (Yukawa *et al.*, 1990). In our own experiments we used salbutamol and found that neither ECP/EDN secretion nor chemiluminescence was substantially affected by this compound. One should mention that according to the experimental protocol used in our studies, cells were treated with salbutamol 10 min prior to the stimulation with C5a; we therefore cannot exclude the possibility that salbutamol would have been effective after a shorter preincubation time diminishing the extent of cyclic AMP degradation by the action of PDE IV. The lack of effect of salbutamol on eosinophil responses is in agreement with the study of Kita *et al.* (1991), who showed no or only marginal inhibitory effects of salbutamol and isoprenaline on sIgA- and IgG-stimulated EDN secretion, respectively, but is in contrast to a recent study of Munoz *et al.* (1994) who demonstrated an approximately 50% inhibition of fMLP-stimulated EPO- (eosinophil peroxidase) and leukotriene C_4-release by salbutamol. The latter group, however, used cytochalasin B as a non-physiological agent in their assays which might facilitate the inhibition of eosinophil responses by β_2-adrenoceptor agonists. In support of this assumption Yukawa *et al.* (1990) failed to find an inhibitory effect of salbutamol on both O_2^- generation and EPO release in eosinophils from patients with eosinophilia. Formoterol at high concentrations (1–100 μmol l^{-1}) was reported to inhibit modestly (<40%)

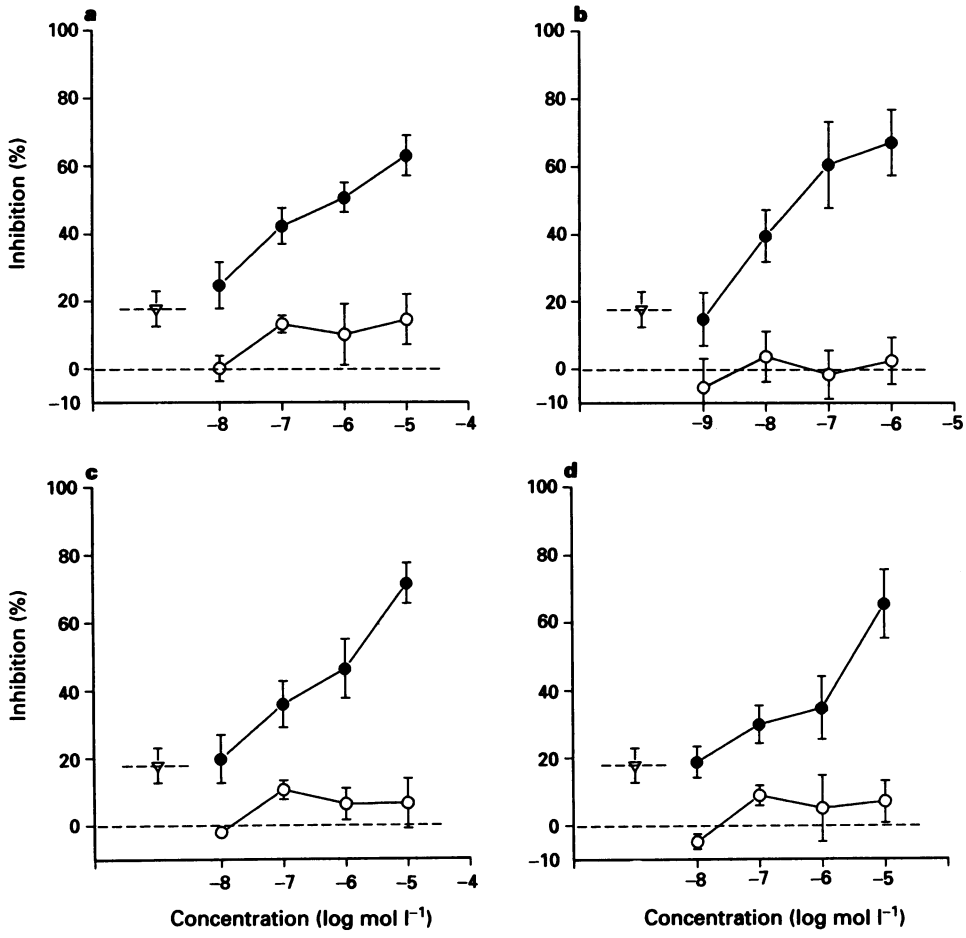

Figure 7 Influence of rolipram (a), RP 73401 (b), zardaverine (c) and tolafentrine (d) (either alone or in combination with 1 μmol l^{-1} salbutamol) on C5a-stimulated chemiluminescence response of human eosinophils. For assay conditions see legend to Figure 6. Symbols refer to the effect of PDE inhibitors alone (○) or in combination with 1 μmol l^{-1} salbutamol (●); the effect of salbutamol itself is also shown (△). Results are given as mean ± s.d. from 3–4 independent experiments.

Table 2 IC_{50} values (μmol l^{-1}) of various PDE inhibitors for inhibition of C5a-stimulated secretion of ECP/EDN and the chemiluminescence response in intact human eosinophils, as well as for inhibition of PDE IV activity in the cytosol of human eosinophils

	Secretion of ECP/EDN		*Chemiluminescence*		*PDE IV activity*
	− Salbut	+ Salbut	− Salbut	+ Salbut	
	IC_{50} (μM)		IC_{50} (μM)		IC_{50} (μM)
Theophylline	589/347	204/257	525	331	288
IBMX	51/50	12/11	524	45	14
Rolipram	NE	0.1/0.2	NE	0.5	0.3
RP 73401	NE	0.05/0.04	NE	0.01	0.0007
Zardaverine	>10	0.7/0.4	NE	0.9	0.3
Tolafentrine	NE	>10	NE	7.6	0.03

Studies of the effect of PDE inhibitors in intact cells were performed either in the absence or presence of salbutamol (Salbut) (1 μmol l^{-1}) which by itself was without appreciable effect. IC_{50} values were calculated from concentration-inhibition-curves by non-linear regression analysis from at least three independent experiments.
NE, no effect. For abbreviations, see text.

PAF-induced ECP release, however, this effect of formoterol was not abolished by propanolol, making a β_2-adrenoceptor-triggered response unlikely (Eda *et al.*, 1993).

Despite the lack of effect of salbutamol itself, this compound allowed the selective PDE inhibitors to inhibit both ECP/EDN secretion and chemiluminescence in a synergistic manner. Obviously, only the cyclic AMP synthesis triggered by the action of the β_2-adrenoceptor agonist and the simultaneous inhibition of cyclic AMP degradation by PDE inhibitors are sufficient to inhibit C5a-stimulated cell responses. Under these conditions the IC_{50} values of rolipram and zardaverine for inhibition of eosinophil responses were in the order of magnitude that would have been expected from the IC_{50} values for inhibition of PDE IV activity in the cell-free system. In contrast, such a conformity did not exist for tolafentrine and RP 73401. Such a poor correlation between the PDE inhibitory actions of certain compounds and their effectiveness in inhibiting O_2^- formation has also been observed in guinea-pig eosinophils and has been attributed to a barrier impeding access of the compounds to the enzyme (Souness *et al.*, 1991). We have tested the compounds used in the present study in other cells and have found no

differences in the ability of the compounds to penetrate into the cells. Alternatively, the aforementioned lack of correlation may be explained by the existence of two sites (a catalytic site and a second biologically important site) on PDE IV as proposed for the guinea-pig eosinophil enzyme (Souness *et al.*, 1992). The second site seems to be (partially) concealed in freshly prepared membranes but is exposed by solubilization or vanadate/glutathione treatment. The IC_{50} values of inhibitors against solubilized PDE IV and their potencies in intact cells (elevation of intracellular cyclic AMP) strongly correlate, implying that in most eosinophils, PDE IV exists in a form similar to the solubilized or vanadate/glutathione-treated enzyme. Although these results obtained with the membrane-bound guinea-pig PDE IV may not directly translate to the cytosolic (soluble) human enzyme, they raise the possibility that the state of human eosinophil PDE IV, including inhibitor-sensitivity in the cytosol, is different from the state of the enzyme in intact cells. We are at present trying to clarify this hypothesis.

Based on the results discussed so far, the question arises why the non-selective PDE inhibitors IBMX and theophylline are able to suppress eosinophil functions in contrast to the selective PDE IV and III/IV inhibitors. The fact that human eosinophils contain PDE IV as the exclusive PDE isoenzyme suggests that other mechanisms besides or in addition to PDE inhibition may contribute to the inhibitory action of IBMX and theophylline. Other modes of action have been described for these xanthine derivatives such as inhibitions of calcium currents (Simasko & Shaochun, 1993), blockade of the adenylate cyclase-inhibitory regulatory protein G_i (Parsons *et al.*, 1988), direct activation of cyclic AMP-dependent protein kinase (Tomes *et al.*, 1993) or, in particular, adenosine receptor antagonism (Ukena *et al.*, 1993). These or other aspects have to be considered in further experiments elucidating the precise mode of action of IBMX and theophylline on human eosinophil functions. At the moment we are able to exclude only adenosine receptor antagonism in the actions of IBMX and theophylline on human eosinophils, based on the fact that human eosinophils have been reported to be very poor producers of adenosine (Resnick *et al.*, 1993) and that the chemiluminescence response is not influenced by adenosine deaminase (data not shown). Nevertheless, the different effects of the PDE inhibitors described in this paper demonstrate that results obtained with non-specific PDE inhibitors may not always translate to specific PDE inhibitors even if the PDE isoenzyme pattern of a certain cell type has been considered as shown here for human eosinophils. Theophylline is a well-established drug in the therapy of airway diseases, although, its mode of action *in vivo* is still controversial (Milgrom & Bender, 1993). Although speculative, the direct inhibitory effect of theophylline on human eosinophil responses observed *in vitro* (Kita *et al.*, 1991; present study) may contribute to the recent clinical finding that after treatment with theophylline a significant reduction in the number of EG2-positive activated eosinophils beneath the lung epithelial basement membrane of mild asthmatics was found (Sullivan *et al.*, 1994). PDE inhibition is ascribed a major role in the action of theophylline on both bronchodilatation and inhibition of inflammation. The benefit arising from theophylline therapy has encouraged the pharmaceutical industry to develop more specific and more potent PDE inhibitors in order to enhance efficacy in parallel with fewer side effects. It will be interesting to see whether such compounds can mimic the action of theophylline in patients suffering from airway diseases. With the restriction that all conclusions are based on experiments using one particular stimulus (C5a), the results presented in this paper indicate that specific PDE IV inhibitors need an additional cyclic AMP-triggering signal to be effective towards eosinophils. We have used a β_2-adrenoceptor agonist (salbutamol) in our *in vitro* experiments; however, it can be assumed that the action of salbutamol is representative of other receptor agonists positively coupled to adenylate cyclase in human eosinophils. Similar to other leukocytes such agonists may include prostaglandins such as PGE_2, adenosine, histamine, endogenous adrenaline and others presumed to be present in inflammatory loci.

In addition, one might speculate whether certain factors (e.g. cytokines, colony-stimulating factors or other inflammatory mediators) present in the circulation and/or in the lung of atopic patients alter the sensitivity of eosinophils towards PDE inhibitors. The study of Maruo *et al.* (1993) may be interpreted in this direction. In that study, an inhibitory effect of the PDE IV inhibitor WAY-PDA-641 on PAF-induced superoxide generation was found in purified eosinophils from allergic subjects, which is in contrast to our own results demonstrating no effect of PDE IV and III/IV inhibitors on C5a-stimulated chemiluminescence in eosinophils from healthy donors. This general aspect is of particular importance for assessing *in vitro*-data on the effects of PDE inhibitors on pro-inflammatory cells for the *in vivo*-efficacy of these drugs in asthmatic patients.

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