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Effects of theophylline and rolipram on leukotriene C_4 (LTC_4) synthesis and chemotaxis of human eosinophils from normal and atopic subjects

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1 The effects of the non-selective phosphodiesterase (PDE) inhibitor theophylline and the selective PDE4 inhibitor rolipram on leukotriene C_4 (LTC_4) synthesis and chemotaxis of complement 5a (C5a)- and platelet-activating factor (PAF)-stimulated human eosinophils obtained from normal and atopic donors were investigated.

2 Eosinophils were purified from peripheral venous blood of normal and atopic subjects by an immunomagnetic procedure to a purity >99%. Eosinophils were stimulated with PAF (0.1 μM) or C5a (0.1 μM) for 15 min and LTC_4 was measured by radioimmunoassay (RIA). Eosinophil chemotaxis in response to PAF and C5a was assessed with 48-well microchambers (Boyden).

3 Under these conditions substantial amounts of LTC_4 (about 300-1000 pg per 10^6 cells) were only detectable in the presence of indomethacin (0.1-10 μM). To explain this finding it was hypothesized that indomethacin reversed the inhibition of LTC_4 synthesis by endogenously synthesized prostaglandins, in particular prostaglandin E_2 (PGE_2). In fact, eosinophils release 23 pg PGE_2 per 10^6 cells following PAF stimulation; this PGE_2 synthesis was completely inhibited by indomethacin and readdition of PGE_2 inhibited eosinophil LTC_4 synthesis (IC_{50}=3 nM). The following experiments were performed in the presence of 10 μM indomethacin.

4 Theophylline (IC_{50}~50 μM) and rolipram (IC_{50}~0.03-0.2 μM) suppressed PAF- and C5a-stimulated LTC_4 synthesis. This PDE inhibitor-induced suppression of LTC_4 generation is mediated by activation of protein kinase A, since it was reversed by the protein kinase A inhibitor Rp-8-Br-cyclic AMPS. In addition, exogenous arachidonic acid concentration-dependently (0.3 μM-3 μM) reversed the inhibition of LTC_4 synthesis by the PDE inhibitors, indicating that theophylline and rolipram suppress the mobilization of arachidonic acid. The β_2-adrenoceptor agonist salbutamol inhibited eosinophil LTC_4 synthesis (IC_{50}=0.08 μM). The combination of salbutamol with theophylline (10 μM) or rolipram (3 nM) appeared to be additive.

5 Theophylline (IC_{50}~40 μM), rolipram (IC_{50}~0.02 μM [C5a], ~0.6 μM [PAF]) and PGE_2 (IC_{50}~3 nM) inhibited C5a- and PAF-stimulated eosinophil chemotaxis. The combination of PGE_2 with theophylline resulted in an additive effect.

6 Both C5a- and PAF-stimulated eosinophil chemotaxis and LTC_4 generation were significantly elevated in eosinophils from atopic individuals compared to normal subjects. However, eosinophils from normal and atopic individuals were not different with respect to their total cyclic AMP-PDE and PDE4 isoenzyme activities as well as the potencies of theophylline and rolipram to suppress LTC_4 generation and chemotaxis.

Keywords: Human eosinophils; C5a; platelet-activating factor (PAF); leukotriene C_4; prostaglandin E_2; chemotaxis; rolipram; theophylline; PDE activity

Introduction

The clinical benefit of theophylline in asthma therapy is based on its bronchodilator and anti-inflammatory effects (Barnes & Pauwels, 1994). Anti-inflammatory actions of theophylline are reflected by its effects on isolated cell function, in animal experiments and under clinical conditions. With respect to isolated cell functions theophylline has been demonstrated to suppress the generation of reactive oxygen radicals (O_2^-) from alveolar macrophages (Dent *et al.*, 1994b), eosinophils (Hatzelmann *et al.*, 1995) and neutrophils (Nielson *et al.*, 1990), eosinophil degranulation (Hatzelmann *et al.*, 1995) and the release of granulocyte-macrophage-colony-stimulating-factor (CM-CSF) from eosinophils (Shute *et al.*, 1995a), monocyte and alveolar macrophage tumour necrosis factor α (TNFα) production (Spatafora *et al.*, 1994), T-lymphocyte chemotaxis (Hidi-Ng *et al.*, 1995) and histamine release from mast cells (Orange *et al.*, 1971; Louis *et al.*, 1992). These anti-inflammatory effects of theophylline *in vitro* are translated clinically to a theophylline-induced improvement of airway inflammation in asthmatics. Theophylline suppressed allergen-induced eosinophil infiltration in the bronchial mucosa (Sullivan *et al.*, 1994) and attenuated the number of T-lymphocytes in the airways of chronic asthmatics (Kidney *et al.*, 1995; Djukanovic *et al.*, 1995). These immunocytochemical findings were paralleled by an improvement of lung function. The anti-inflammatory and bronchodilator effects of theophylline may originate in an inhibition of cyclic 3':5' monophosphate phosphodiesterases (PDEs). Therefore, the anti-asthmatic clinical profile of theophylline has prompted the search for new, selective PDE inhibitors for asthma therapy. PDEs hydrolyse the cyclic nucleotides adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) and, as a consequence, PDE inhibition results in an intracellular increase of cyclic nucleotide levels and an elevation of cyclic AMP- and cyclic GMP-dependent protein kinase activity. Increased cyclic nucleotide concentrations may inhibit inflammatory cell functions and attenuate smooth muscle tone.

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The PDEs comprise at least 7 isoenzymes (PDE1–7 according to the nomenclature by Beavo & Reifsnyder (1990)). PDE4–the rolipram-sensitive cyclic AMP-specific PDE has been identified as the predominant PDE isoenzyme in many inflammatory cells such as eosinophils, monocytes, neutrophils (Barnes, 1995; Schudt *et al.*, 1995). By use of RT-PCR four PDE4 subtypes have been identified (PDE4A-D) and these subtypes are subject to cyclic AMP-dependent regulation by enzyme induction and protein phosphorylation (Conti *et al.*, 1995). The occurrence of PDE4 in inflammatory cells gave rise to the development of selective PDE4 inhibitors and the need to evaluate their anti-inflammatory potency. A multitude of such compounds have been synthesized and the clinical development of CDP 840, RP 73401, LAS 31025, Way PDA-641, SB 207499 (Palfreyman, 1995) as anti-asthmatic drugs is currently pursued. Most of these compounds share the 3-cyclopentyloxy-4-methoxy-phenyl moiety of rolipram which represents the archetypic selective PDE4 inhibitor. Rolipram (<10 μM) has been used in many studies to investigate effects of PDE4 inhibition on cellular functions. An important cellular target of PDE4 inhibitors in asthma is represented by eosinophils not only because airway eosinophilia is a feature of asthma (Bousquet *et al.*, 1990), but also because eosinophils release a series of inflammatory mediators (cytotoxic O_2^-, eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), major basic protein (MBP) and bronchoconstrictor cysteinyl leukotrienes). Eosinophils exclusively contain PDE4 and PDE4 inhibitors have been found to suppress eosinophil functions such as O_2^--generation and degranulation of cationic proteins (Dent *et al.*, 1991; 1994a; Souness *et al.*, 1991; Hatzelmann *et al.*, 1995).

The purpose of this study was to investigate the effects of the nonselective PDE inhibitor theophylline and the selective PDE4 inhibitor rolipram on human eosinophil leukotriene C_4 (LTC_4) synthesis and chemotaxis induced by two different stimuli (complement 5a (C5a) and platelet activating factor (PAF)). These two parameters were considered to be of particular importance. Firstly, inhibition of LTC_4 synthesis could provide a mechanism for PDE inhibitor-induced indirect bronchodilatation since PAF-stimulated eosinophils have been shown to induce leukotriene-dependent bronchoconstriction (Rabe *et al.*, 1994). An inhibitory effect of theophylline on leukocyte LTC_4 generation has recently been noted by Kristiansson *et al.* (1994). Secondly, suppression of eosinophil chemotaxis *in vitro* would provide a rationale for the inhibition of eosinophilic airway inflammation by PDE inhibitors *in vivo* (Teixera *et al.*, 1994; Banner & Page 1995). Recently, it has been demonstrated that cyclic AMP-elevating agents, including the selective PDE4 inhibitors RS 25344 (Kaneko *et al.*, 1995) and Way PDA-641 (Tanimoto *et al.*, 1994), may inhibit eosinophil migration *in vitro*. Moreover, in view of the finding that PDE activities in monocytes from subjects with atopic dermatitis were elevated when compared with normals (Butler *et al.*, 1983; Chan & Hanifin 1993; Chan *et al.*, 1993b), eosinophils from normal and atopic individuals were compared with respect to their total cyclic AMP-PDE and PDE4 activities and the effects of theophylline and rolipram on cell functions. Preliminary data of the chemotaxis work have been recently published as conference reports (Shute *et al.*, 1994; Schudt *et al.*, 1995).

Methods

Isolation of human eosinophils

Peripheral venous blood (100 ml) was obtained from normal and atopic donors and anticoagulated with heparin (10 u ml^{-1}). Blood was diluted with an identical volume of PBS/FCS consisting of phosphate buffered saline (PBS; $mmol^{-1}$: KCl 2.7, NaCl 137, Na_2HPO_4 8.1, KH_2PO_4 1.5, pH 7.4) supplemented with heat inactivated foetal calf serum (FCS) to a final concentration of 2%. Diluted blood (36 ml) was carefully layered on top of 18 ml isotonic percoll (density 1.082 g ml^{-1}) and centrifuged at 600 *g* for 30 min at room temperature. To prepare isotonic percoll, 100% percoll was diluted with HEPES/HBSS consisting of Hank's balanced salt solution (HBSS) supplemented with N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (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 density of 1.082. Following centrifugation, the upper layer containing mononuclear cells was discarded and the red cell pellet containing the polymorphonuclear cells (PMN) was carefully removed and placed into another tube. Red cells were lysed for 15 min at 4°C in a buffer containing, mmol l^{-1}. NH_4Cl 155, $KHCO_3$ 10 and ethylenediaminetetraacetic acid (EDTA) 0.1; pH 7.4. The resulting PMN were washed twice with PBS/FCS (150 *g* for 5 min at 4°C) and finally resuspended in 500 μl PBS/FCS; 100 μl of anti-CD16 MACS-microbeads were added and the cells were left on ice for 30 min to allow neutrophils to become labelled by the anti-CD16 antibodies. To separate unlabelled eosinophils from magnetically labelled neutrophils, PMN were layered on top of a MACS-column type C which was placed inside a strong magnetic field. Unlabelled eosinophils were eluted with 25 ml PBS/FCS whereas magnetically labelled neutrophils were retained on the column due to a strong magnetic field. This procedure resulted in about 95%–97% pure eosinophils with neutrophils as contaminating cells. To further increase the purity of eosinophils the immunomagnetic procedure was repeated with the exceptions that 30 μl anti-CD16 MACS-microbeads were added to the cell suspension followed by cell separation on a MACS-column type B2. The purity of eosinophils attained >99.5% as assessed by Kimura staining after this second procedure. Viability of eosinophils was >97% as measured by trypan blue exclusion. The number of eosinophils isolated from 100 ml blood from normal and atopic donors was $1.2 \pm 0.1 \times 10^7$ cells and $3.1 \pm 0.5 \times 10^7$ cells, respectively ($P = 0.02$).

Eosinophil LTC_4 and prostaglandin E_2 synthesis

Freshly prepared eosinophils were resuspended in HEPES/HBSS with calcium and magnesium (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, Ca 1, Mg 1, HEPES 10; pH 7.4) supplemented with 0.1% gelatine and 20 mmol l^{-1} L-serine to a final concentration of 10^6 cells and 2×10^6 cells in 200 μl incubation volume for LTC_4 and prostaglandin E_2 (PGE_2) assays, respectively. Eosinophils were preincubated with the test compounds as indicated for 10 min at 37°C. Thereafter, cells were stimulated for 15 min at 37°C with PAF or C5a as indicated in the legends to the Figures. LTC_4 and PGE_2 synthesis was terminated by adding ethanol to a final concentration of 66%. The incubation mixture was left in an ice-water bath for 1 h, centrifuged at 1000 *g* for 15 min and the supernatants were stored at −70°C for analysis of arachidonic acid metabolites. LTC_4 and PGE_2 were measured by commercially available radioimmunoassays (RIAs) following the instructions of the manufacturer (assay range: 12.5–800 pg LTC_4 per tube; 0.15–160 pg PGE_2 per tube).

Eosinophil chemotaxis

Eosinophil chemotaxis was assessed with 48-well microchambers (Boyden). The volumes of the wells in the lower and the upper chambers were 25 μl and 50 μl, respectively. The lower and the upper chamber were separated by a filter (PVP free; 8 μM). Eosinophils and test compounds were suspended in chemotaxis buffer (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, Ca 1, Mg 1, supplemented with 0.1% bovine serum albumin (BSA)). The wells of the lower chamber contained chemotactic agents (C5a and PAF) and test compounds and the wells of the upper chamber contained eosinophils (10^6 cells

ml^{-1}) and test compounds. Chemotaxis chambers were incubated at 37°C to allow eosinophil chemotaxis. A positive chemotaxis response was defined as the migration of eosinophils through the filter and adherence to the lower side. After 1 h incubation, filters were removed and non-migrated cells were scraped from the upper side of the filter. The filters were stained (Haema Gurr) and the number of migrated eosinophils was counted. Results are given as chemotactic index (C.I.), which is defined as the ratio of the number of cells migrated through the filter in the presence and absence of chemotactic agents, or as % inhibition of control.

Measurement of PDE activity

PDE activity from freshly prepared human eosinophils was measured under cell-free conditions. Eosinophils (4×10^6) were centrifuged at 150 *g* for 5 min and resuspended in 1 ml homogenization buffer (composition, mmol l^{-1}: KCl 2.7, NaCl 137, Na_2HPO_4 8.1, KH_2PO_4 1.5, HEPES 10, EDTA 1, $MgCl_2$ 1, β-mercaptoethanol 1, pH 8.2). Cells were disrupted by sonication (Branson sonifier; step 2; 2×30 s with a 15 s break) which resulted in >98% of disrupted cells as assessed by trypan blue exclusion.

PDE IV activity was immediately measured in the complete homogenates by the method of Thompson & Appleman (1979) with some modifications (Bauer & Schwabe 1980). The assay mixture (final volume 200 μl) contained Tris HCl 40 mM (pH 7.4), $MgCl_2$ 5 mM, cyclic AMP 0.5 μM including [^{3}H]-cyclic AMP (about 50000 c.p.m. per assay), either 10 μM rolipram or vehicle and 50 μl eosinophil homogenate corresponding to 200000 cells. Incubations were for 15 min at 37°C and reactions were stopped by adding 50 μl 0.3 N HCl. Assays were left on ice for 10 min and then 25 μg 5'-nucleotidase (Crotalus atrox snake venom) was added. Following incubation for 20 min at 37°C, the assay mixtures were loaded on top of QAE-Sephadex A25 columns (bed volume 1 ml) and [^{3}H]-adenosine was eluted from the column with 2 ml 30 mM ammonium formate pH 6.0. The amount of radioactivity in the eluate was counted. Results were corrected for blank values (measured in presence of denatured protein) which did not exceed 2% of the total radioactivity. The amount of cyclic AMP hydrolysed did not exceed 20% of the original substrate concentration. Rolipram was dissolved in 0.1% dimethyl sulphoxyde (DMSO) (final concentration). PDE4 activity was defined as the difference of PDE activity measured in the presence and absence of 10 μM rolipram; 10 μM rolipram has been demonstrated to inhibit almost completely PDE4 activity (Tenor *et al.*, 1995).

Patients

Blood was taken from atopic and non-atopic volunteers between 07 h 00 min and 09 h 00 min. Atopy was assessed by positive skin tests to one or more common allergens and serum IgE > 80 u ml^{-1}. Atopic subjects were usually free of symptoms but had a history of allergic rhinitis, mild asthma or mild atopic dermatitis. At the time of the investigations, subjects were free of medications. The study was approved by the local ethics committee.

Chemicals and solutions

PAF (platelet activating factor), C5a (complement factor 5a), EGTA (ethyleneglycol-bis-[β-aminoethylether]N,N,N',N'-tetra acetic acid), theophylline, serine, gelatine, *Crotalus atrox* snake venom, percoll, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]); EDTA (ethylene diamine tetraacetic acid), β-mercaptoethanol, cyclic AMP, salbutamol, arachidonic acid, prostaglandin E_2, PGF_{2a} and PGD_2, indomethacin were purchased from Sigma Ltd, Poole, Dorset, U.K. The protein kinase A activator Sp-5.6-cBIMPS (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole 3',5' cyclic mono-phosphorothioate, Sp-isomere) and the protein kinase A inhibitor Rp-8-Br-cyclic AMPS (8-bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomere) were from Biolog, Bremen, Germany. PBS, HBSS and heat inactivated foetal calf serum (FCS; E.U. approved) were obtained from Gibco Life Sciences BRL, Paisley, Scotland, U.K. Anti-CD16 MACS-microbeads, MACS-columns type C and B2 and the MACS magnet were purchased from Miltenyi Biotec, Bergisch Gladbach, Germany. [^{3}H]-cyclicAMP, the leukotriene $C_4/D_4/E_4$ ^{3}H-RIA assay system and the prostaglandin E_2 ^{125}I-RIA assay system were from Amersham International plc, Buckinghamshire, U.K. Haema Gurr - Staining solutions and all other chemicals and solvents were of analytical grade and were obtained from BDH Laboratory Supplies Ltd, Poole, Dorset, U.K. Rolipram was a generous gift from Schering AG (Berlin, Germany). Microchemotaxis chambers (48 well) were purchased from Neuro Probe Inc, Cabin John, MD, U.S.A. Membrane filters (PVP free; 8 μM) were obtained from Nucleopore GmbH, Tuebingen, Germany.

Statistical analysis of data

Results are shown as mean ± s.e.mean from the number (*n*) of independent experiments indicated. IC_{50} values were calculated by the InPlot program (GraphPad Software Inc, Philadelphia, U.S.A.). Statistical significance was determined by Student's *t* test (InStat from Graphpad Software Inc, Philadelphia, U.S.A.). Statistical significance was defined as $P < 0.05$ (*).

Results

Eosinophil LTC_4 synthesis

Initial experiments were performed to assess the ability of 0.1 μM PAF and 0.1 μM C5a to stimulate LTC_4 synthesis in eosinophils obtained from normal and atopic donors. As shown in Figure 1, LTC_4 synthesis following incubation with PAF and C5a alone was hardly detectable. Since previous results from Kuehl *et al.* (1984) indicated that indomethacin enhanced neutrophil LTB_4 synthesis we questioned whether the cyclo-oxygenase inhibitor could give rise to the generation of substantial amounts of LTC_4 from C5a- and PAF-stimulated eosinophils. In fact, indomethacin concentration-dependently enhanced PAF- and C5a-stimulated eosinophil LTC_4 synthesis. In the presence of 10 μM indomethacin 0.1 μM PAF more potently stimulated LTC_4 synthesis than 0.1 μM C5a. Furthermore, PAF- and C5a-stimulated eosinophils from atopics generated significantly more LTC_4 than eosinophils from normals (Figure 1). Indomethacin (10 μM) in the absence of PAF or C5a did not affect eosinophil LTC_4 synthesis. Considering the mechanism of the indomethacin-induced increase of PAF- and C5a-stimulated LTC_4 synthesis in eosinophils we hypothesized that endogenous eosinophil-derived prostanoids (e.g. PGE_2) inhibit LTC_4 synthesis and, consequently, that this inhibition might be abolished by indomethacin. Several lines of evidence support this hypothesis. Firstly, PGE_2 production was induced by PAF to a mean of 23 pg PGE_2 per 10^6 cells which was completely inhibited by 10 μM indomethacin. Secondly, readdition of PGE_2 in the presence of indomethacin suppressed LTC_4 synthesis ($IC_{50} = 2$ nM). PGE_2 was 100 fold more potent than $PGF_{2\alpha}$ and PGD_2 in this respect (Figure 2). Thirdly, a protein kinase A (PKA)-inhibitor (Rp-8-Br-cyclic AMPS, PKA-I), which is suggested to block the PGE_2-mediated effect, potentiated PAF-stimulated LTC_4 synthesis to a similar extent as indomethacin (Figure 3).

To ensure measurable amounts of LTC_4, subsequent experiments were performed in the presence of 10 μM indomethacin. Time-course experiments revealed that a 15 min LTC_4 generation had reached a plateau. From concentration-response experiments 0.1 μM PAF and 0.1 μM C5a were selected as stimulus concentrations.

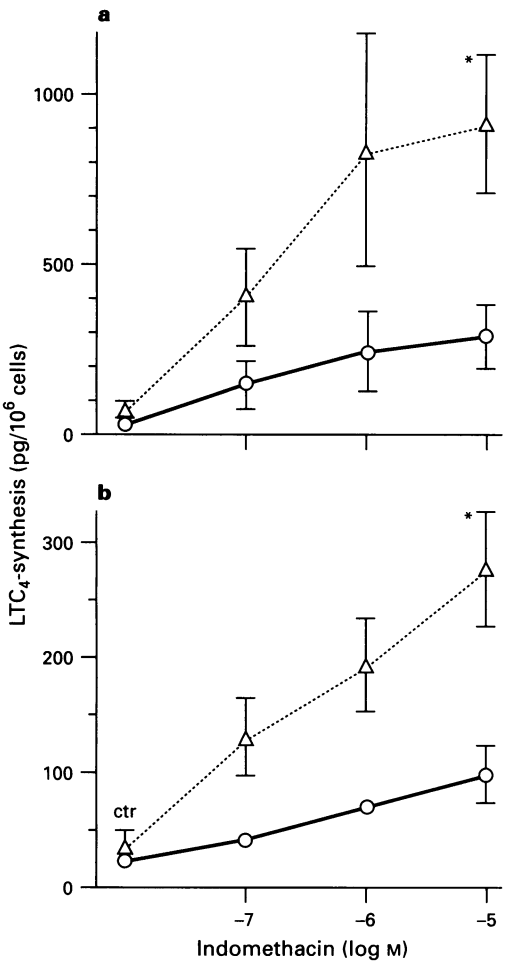

Figure 1 The effect of indomethacin on (a) PAF- and (b) C5a-stimulated LTC_4 generation of human eosinophils from normal (○) and atopic (△) individuals. Human eosinophils (10^6 cells $200\,\mu l^{-1}$) were preincubated with indomethacin ($0.1\,\mu M - 10\,\mu M$) for 10 min and stimulated with C5a ($0.1\,\mu M$) or PAF ($0.1\,\mu M$) for 15 min. Incubations were terminated by adding ice-cold ethanol to a final concentration of 66% and LTC_4 was measured by RIA. Data are given as mean ± s.e.mean from 6–10 experiments. Statistical significance ($^*P<0.05$) by t tests was calculated by comparing LTC_4 generation from normal and atopic individuals.

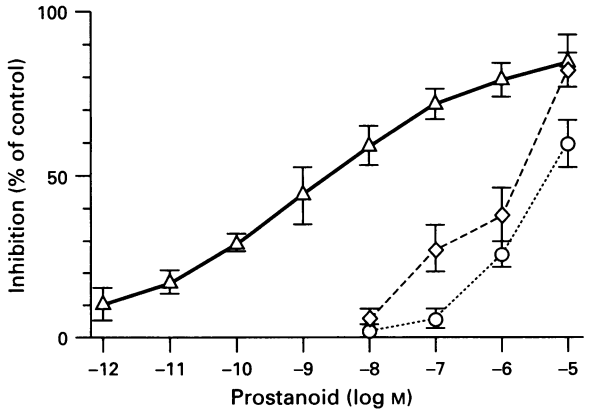

Figure 2 Effects of prostanoids on human eosinophil LTC_4 generation. Human eosinophils (10^6 cells $200\,\mu l^{-1}$) were preincubated with PGE_2 ($10^{-12} - 10^{-5}$ M; △), PGD_2 (◇) or $PGF_{2\alpha}$ ($10^{-8} - 10^{-5}$ M; ○) and $10\,\mu M$ indomethacin for 10 min and stimulated with PAF ($0.1\,\mu M$). After 15 min, incubations were terminated by addition of ethanol and LTC_4 was measured by RIA. Each data point represents the mean from 3 experiments; vertical lines show s.e.mean.

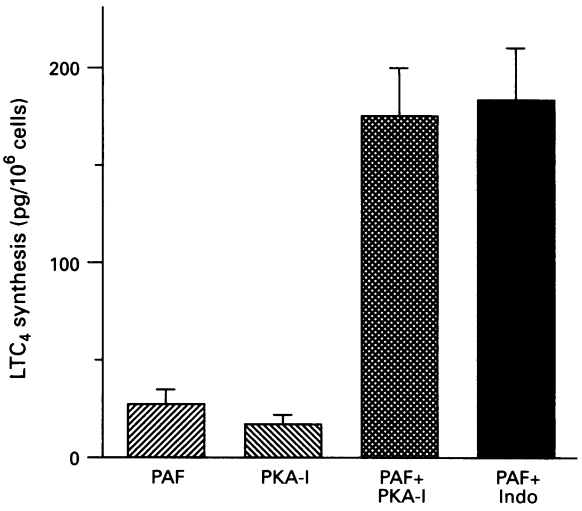

Figure 3 Effect of a protein kinase A inhibitor on PAF-induced eosinophil LTC_4 generation. Human eosinophils (10^6 cells $200\,\mu l^{-1}$) were preincubated with either $100\,\mu M$ Rp-8-Br-cyclic AMPS (protein kinase A inhibitor; PKA-I), $10\,\mu M$ indomethacin (Indo) or vehicle and stimulated with $0.1\,\mu M$ PAF or vehicle. After 15 min, the incubations were terminated and LTC_4 was measured by RIA. Results are given as mean from $n=4$ experiments; vertical lines shown s.e.mean.

Inhibition of LTC_4 synthesis by PDE inhibitors

Theophylline and rolipram concentration-dependently inhibited PAF- and C5a-stimulated LTC_4) synthesis (Figure 4). IC_{50} values are shown in Table 1. Rolipram was approximately 600 fold more potent than theophylline in suppressing LTC_4 synthesis. The efficacy of rolipram and theophylline in inhibiting LTC_4 synthesis of eosinophils from normal and atopic individuals or following stimulation of eosinophils with PAF and C5a was found to be not significantly different. Other cyclic AMP-elevating agonists such as the β_2-adrenoceptor agonist salbutamol also inhibited PAF- or C5a-stimulated LTC_4 synthesis ($IC_{50}=0.08\,\mu M$); its combination with $10\,\mu M$ theophylline or 3 nM rolipram was additive (data not shown). Figure 5 illustrates that the PKA-activator (PK-A) 5.6DClcBIMPS completely inhibited PAF-induced LTC_4 synthesis. In contrast, the PKA-inhibitor (PKA-I) Rp-8-Br-cyclic AMPS largely reversed the inhibition of LTC_4 synthesis by theophylline and rolipram. Although we did not investigate the exact mechanism by which PDE inhibitors suppress LTC_4 synthesis in detail, preliminary experiments suggested that theophylline and rolipram inhibited the mobilization of arachidonic acid since the inhibition of LTC_4 generation by these compounds was concentration-dependently reversed by exogenous arachidonic acid ($0.3\,\mu M - 3\,\mu M$).

Eosinophil chemotaxis

C5a and PAF concentration-dependently triggered eosinophil chemotaxis. Concentration-response curves for C5a and PAF were bell-shaped. This finding is illustrated for C5a in Figure 6. The maximally effective concentrations of 1 nM C5a and 100 nM PAF were used in subsequent experiments. The chemotactic index (defined as the ratio of cells migrating in the presence and absence of a chemotactic gradient) found for PAF- or C5a-stimulated eosinophils from atopic individuals were significantly higher than those from normal subjects.

Rolipram and theophylline inhibited C5a- (Figure 7; Table 1) and PAF-induced (Table 1) (Schudt *et al.*, 1995) eosinophil chemotaxis. Theophylline suppressed C5a- and PAF-induced eosinophil chemotaxis from normal and atopic subjects with similar potency. Rolipram equi-effectively inhibited chemotaxis of eosinophils from normal and atopic subjects but was approximately 10–30 fold more potent in suppressing C5a-

induced chemotaxis than PAF-induced chemotaxis. In comparison to theophylline, concentration-inhibition curves obtained for rolipram were more flat (Figure 7).

Besides PDE inhibitors, PGE_2 was demonstrated to suppress C5a-induced eosinophil chemotaxis ($IC_{50} = 3$ nM) and its combination with theophylline (10 μM) resulted in an additive effect. Eosinophil chemotaxis was also significantly attenuated by 0.1 μM salbutamol (55% inhibition) and 100 μM 5.6DCIc-BIMPS, a protein kinase A activator (80% inhibition). In addition, theophylline-induced inhibition of eosinophil chemotaxis was reversed by the PKA-inhibitor Rp-8-Br-cyclic AMPS by about 70%.

Eosinophil PDE IV activity

It has recently been demonstrated that human eosinophils predominantly contain soluble PDE4 (Hatzelmann *et al.*, 1995). These studies were extended by investigating whether total cyclic AMP-PDE activities and PDE4 activities from homogenates of normal and atopic donors were different. For this purpose eosinophils from 6 normal and 6 atopic individuals were homogenized and analysed for PDE4 activity under identical experimental conditions (see Methods for details). As shown in Table 2, total and PDE4 activities of normal and atopic asthmatic subjects were not significantly different. In addition, rolipram and theophylline inhibited PDE activities from eosinophils obtained from normal and atopic individuals with similar potencies as reflected by the IC_{50} values given in Table 2.

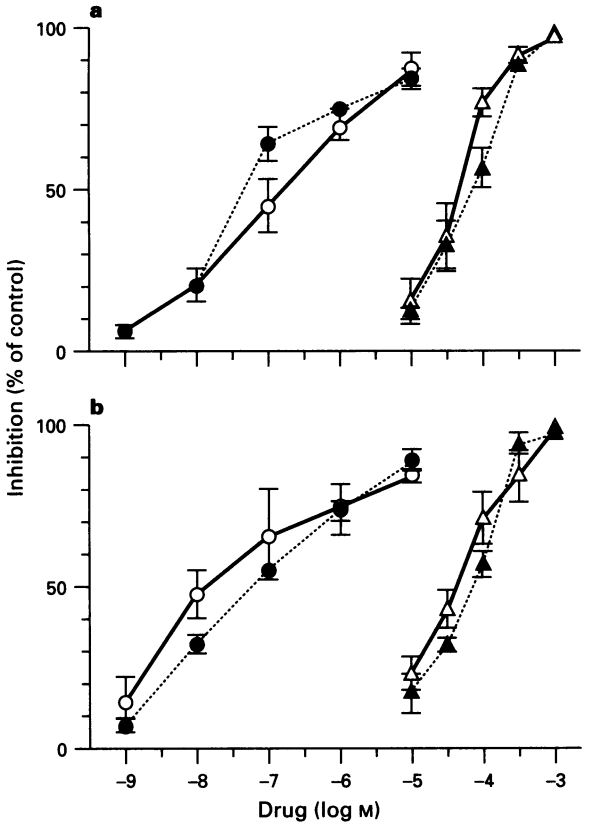

Figure 4 Effect of theophylline and rolipram on (a) PAF- and (b) C5a-stimulated human eosinophil LTC_4 generation. Human eosinophils (10^6 cells 200 μl^{-1}) were obtained from normal (open symbols) and atopic (solid symbols) subjects and preincubated with theophylline (10^{-5}–10^{-3} M; triangles) or rolipram (10^{-9}–10^{-5} M; circles) in the presence of 10 μM indomethacin. Following incubation with either 0.1 μM PAF or 0.1 μM C5a for 15 min, LTC_4 was measured in ethanolic extracts. Results represent the mean ± s.e.mean from at least 4 experiments.

Discussion

In this study, we investigated effects of the non-selective PDE inhibitor theophylline and the selective PDE4 inhibitor rolipram on human eosinophil LTC_4 synthesis and chemotaxis induced by PAF and C5a. We found that both compounds concentration-dependently inhibited eosinophil chemotaxis and LTC_4 synthesis. The inhibitory potency of theophylline and rolipram was similar with respect to the functional responses analysed (chemotaxis and LTC_4 generation) and the stimuli used (PAF and C5a) (Table 1). Furthermore our results suggest that the potent inhibition of eosinophil LTC_4 synthesis and chemotaxis by theophylline with an IC_{50} of about 50 μM (9 $\mu g\ ml^{-1}$), which corresponds

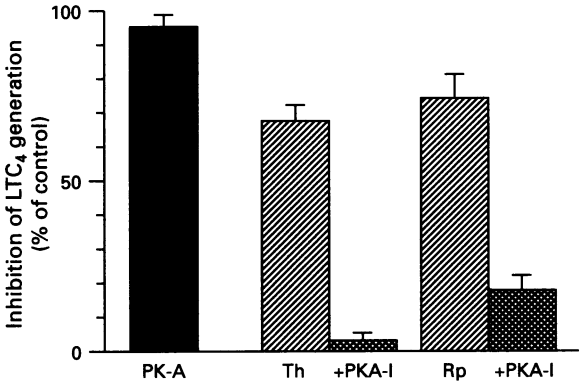

Figure 5 The effects of a protein kinase A-activator on eosinophil LTC_4 synthesis and of a protein kinase A-inhibitor on the suppression of LTC_4 synthesis by theophylline or rolipram. Human eosinophils (10^6 cells 200 μl^{-1}) were preincubated for 30 min with 1 mM Rp-8-Br-cyclic AMPS (protein kinase A-inhibitor; PKA-I) or vehicle and for 10 min with 100 μM 5.6DClcBIMPS (protein kinase A-activator; PK-A), 100 μM theophylline (Th) or 1 μM rolipram (Rp) and with 10 μM indomethacin. Eosinophils were stimulated with 0.1 μM PAF for 15 min and LTC_4 was measured for RIA. Results are given as mean ± s.e.mean from 3 experiments.

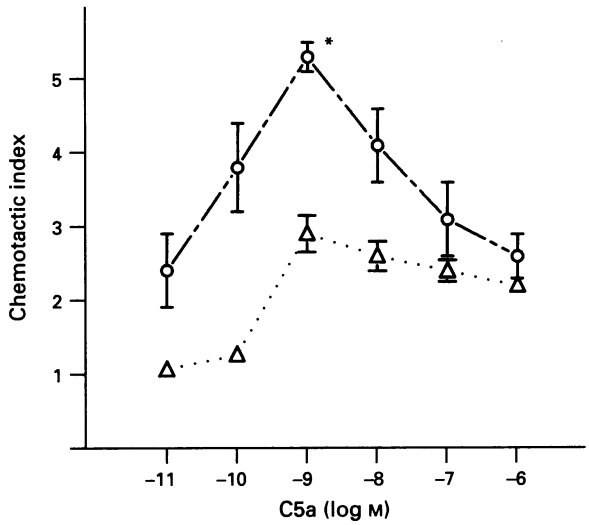

Figure 6 C5a-stimulated chemotaxis of human eosinophils from normal (△) and atopic (○) subjects. Human eosinophils (10^6 cells ml^{-1}; upper wells) and C5a (10^{-11}–10^{-6} M; lower wells) were placed into 48 well microchambers. Chambers were separated by a filter (PVP-free; 8 μM) and incubated for 1 h at 37°C. Filters were stained (Heama-Gurr) and migrated cells were counted by light microscopy. Results are expressed as chemotactic index, which represent the ratio of migrated cells in the presence or absence of C5a. Data are given as mean ± s.e.mean from 5 experiments. Statistical significance ($^*P < 0.05$) by t tests was calculated when comparing chemotaxis from normal and atopic individuals.

Table 1 IC_{50} values (μM) of rolipram and theophylline for inhibition of PAF- and C5a-stimulated LTC_4 generation and chemotaxis of human eosinophils isolated from normal and atopic subjects

	PAF		*C5a*	
	Normal	*Atopic*	*Normal*	*Atopic*
	IC_{50} (μM)		*IC_{50} (μM)*	
	LTC_4 generation			
Rolipram	0.2±0.08	0.08±0.02	0.03±0.01	0.08±0.01
Theophylline	50±3.1	63±4.7	39±2.8	63±7.2
	Chemotaxis			
Rolipram	0.6±0.1	0.55±0.15	0.02±0.005	0.04±0.01
Theophylline	43±8.4	37±4.8	39±6.9	70±13.2

IC_{50} values were calculated by non-linear regression from concentration-inhibition curves and are given as mean±s.e.mean from at least 4 experiments.

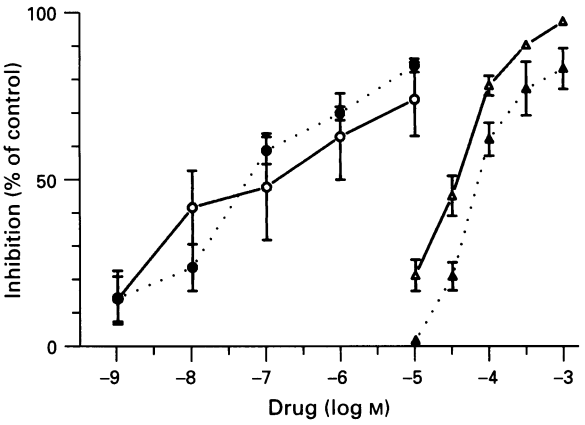

Figure 7 The effects of rolipram (circles) and theophylline (triangles) on C5a-stimulated chemotaxis of human eosinophils obtained from normal (open symbols) and atopic (solid symbols) subjects. C5a (1 nM) and rolipram ($10^{-9}-10^{-5}$ M) or theophylline ($10^{-5}-10^{-5}$ M) were placed into the lower wells and eosinophils (10^6 cells ml^{-1}) and identical concentrations of rolipram or theophylline were placed into the upper wells of 48 well microchambers. The upper and the lower wells were separated by a filter (8 μM; PVP-free) and the chambers were incubated at 37°C for 60 min. Thereafter migrated cells were counted. Results represent the mean from 4–9 experiments; vertical lines show s.e.mean.

to the recommended theophylline serum concentration under clinical conditions (5–15 $\mu g\ ml^{-1}$), might reflect a clinically relevant anti-inflammatory effect of this xanthine. A comparison of the effects of theophylline and rolipram on C5a-stimulated eosinophil LTC_4 synthesis and chemotaxis as described in this study and on C5a-stimulated eosinophil degranulation and O_2^--generation (Hatzelmann *et al.*, 1995) revealed that PDE inhibitors suppress eosinophil functions differentially. In contrast to our results, C5a-induced degranulation and O_2^--generation were not affected by rolipram and only weakly inhibited by theophylline (IC_{50} = 350–540 μM). In addition, effects of PDE inhibitors on eosinophil functions varied with respect to the stimulus used. Whereas rolipram potently attenuated C5a-stimulated eosinophil chemotaxis, the selective PDE4 inhibitor did not affect IL5-stimulated chemotaxis (Shute *et al.*, 1994; Schudt *et al.*, 1995). Taken together, these data suggest that signal transduction by different stimuli (PAF, C5a, IL5) and different functions (e.g. LTC_4, chemotaxis, O_2^-, degranulation) are differentially affected by PDE inhibitors. In similar studies Kaneko *et al.* (1995) demonstrated that the selective PDE4 inhibitor RS25344 significantly attenuated C5a- and PAF-induced human eosinophil chemotaxis. However, maximal inhibition of chemotaxis with RS25344 attained only 40% whereas maximal inhibition with rolipram was 70–80% and with theophylline 80–90%.

Table 2 Total cyclic AMP-PDE activities, PDE4 activities and IC_{50} values of rolipram and theophylline for inhibition of PDE activities in homogenates of human eosinophils isolated from normal and atopic individuals

	Normal	*Atopic*
Cyclic AMP-PDE activity (pmol min^{-1} per 10^8 cells	250±5	291±18
PDE IV activity (pmol min^{-1} per 10^8 cells)	218±5	246±14
Inhibition of PDE4 (IC_{50}; μM)		
Rolipram	0.2	0.16
Theophylline	265	251

Total cyclic AMP-PDE activities were measured at 0.5 μM cyclic AMP substrate concentration. PDE4 activities represented the rolipram (10 μM) inhibitable portion of total cyclic AMP-PDE activity. Results are mean±s.e.mean from 6 independent experiments. IC_{50} values were calculated from concentration-inhibition curves by non-linear regression and are given as mean from 3 experiments.

In view of recent data demonstrating that PDE4 inhibitors are more potent in suppressing proliferation (Banner *et al.*, 1995) and IL4-synthesis (Chan *et al.*, 1993a) by mononuclear cells obtained from atopic subjects than from normal individuals, we have compared the effects of rolipram and theophylline on LTC_4 synthesis and chemotaxis of eosinophils obtained from normal and atopic individuals. As shown in Table 1, eosinophil functions were suppressed by the PDE inhibitors in a similar fashion. These results correspond to the fact that total PDE activity, PDE4 activity and the potency of rolipram and theophylline in inhibiting cyclic AMP-PDE activity were not different between eosinophils from normal and atopic subjects (Table 2). These data are in contrast to the data from Butler *et al.* (1983), Chan & Hanifin (1993) and Chan *et al.* (1993b) which showed that PDE activities in mononuclear cells from atopic individuals were elevated compared to normals. To explain this discrepancy it may be hypothesized, that a cytokine-driven change of PDE activities occurs in peripheral blood mononuclear cells (as suggested by Li *et al.* (1992)) but not in eosinophils from atopic subjects. In parallel to the findings with eosinophils, PDE isoenzyme activity profiles from alveolar macrophages obtained from normal and atopic subjects were not different (Tenor *et al.*, 1995).

Theophylline and rolipram are considered to mediate their effects by an elevation of cyclic AMP and subsequent activation of protein kinase A. Therefore, we investigated whether other cyclic AMP-elevating agents affect eosinophil LTC_4 synthesis and chemotaxis. The β_2-adrenoceptor agonist salbutamol and PGE_2 were shown to suppress potently chemotaxis and LTC_4 generation. However, since their combination with theophylline and rolipram resulted in an additive rather

than a synergistic effect, a cyclic AMP-independent action of salbutamol and PGE_2 cannot be excluded. In contrast to our results, the combination of salbutamol with rolipram and theophylline synergistically enhanced the suppression of C5a-stimulated eosinophil degranulation and O_2^--release (Hatzelmann *et al.*, 1995). In order to test whether activation of protein kinase A mediates the inhibition of LTC_4 generation and chemotaxis by theophylline and rolipram, the effects of the protein kinase A activator 5.6DClcBIMPS and the protein kinase A inhibitor Rp-8-Br-cyclic AMPS on these eosinophil functions were investigated. The protein kinase A activator abolished LTC_4 synthesis and chemotaxis indicating that activation of protein kinase A represents a possible pathway by which these eosinophil functions can be blocked. In addition, the protein kinase A inhibitor reversed the inhibition of LTC_4 synthesis and chemotaxis by the PDE inhibitors. These results suggest that the effects of theophylline and rolipram on eosinophil LTC_4 generation and chemotaxis were mediated by activation of protein kinase A.

Although the potency of theophylline and rolipram in inhibiting the functional response of eosinophils from normal and atopic subjects was similar, C5a- and PAF-stimulated cells from atopic individuals exhibited an elevated LTC_4 generation and chemotactic response when compared to cells from normal subjects. These findings are confirmed by data demonstrating that A23187-stimulated eosinophil LTC_4 synthesis in atopic subjects is higher than in normal individuals (Taniguchi *et al.*, 1985; Wang *et al.*, 1989; Aizawa *et al.*, 1990). This elevated LTC_4 generation and chemotactic response might originate in the 'priming' of eosinophils from atopic subjects by cytokines such as IL3 and IL5. These cytokines have been recently demonstrated to enhance substantially PAF-stimulated eosinophil LTC_4 generation (Takafuji *et al.*, 1991) and chemotaxis (Hakansson & Venge, 1994).

The effects of PDE inhibitors on PAF- and C5a-stimulated eosinophil LTC_4 synthesis were investigated in the presence of 10 μM indomethacin. Indomethacin concentration-dependently enhanced PAF- and C5a-stimulated eosinophil LTC_4 generation (Figure 1). The rationale for the use of indomethacin was two fold: Firstly, in the absence of indomethacin, PAF- and C5-stimulated LTC_4 synthesis was too small for generating reliable concentration-response curves for PDE inhibitors and secondly, an enhancement of neutrophil LTB_4 synthesis by indomethacin has been obtained previously (Keuhl *et al.*, 1984). In addition, inclusion of indomethacin served to exclude effects of endogenous prostanoids on the potency of theophylline and rolipram to suppress eosinophil LTC_4 synthesis. Our observation that in the absence of indomethacin PAF <1 μM and C5a <0.1 μM induced very small amounts of LTC_4 is in agreement with recent findings by Takafuji *et al.* (1991), Kok *et al.* (1989) and Bruynzeel *et al.* (1986). In contrast to these studies, Tamura *et al.* (1988) detected substantial PAF (0.1 μM)-induced eosinophil LTC_4 synthesis (200 pg per 10^6 cells) in the absence of indomethacin. The reason for the different results from Tamura *et al.* (1988) cannot be easily explained. It is possible that neutrophil contamination of eosinophil preparations may account for the substantial PAF-induced eosinophil LTC_4 generation in the study from Tamura *et al.* (1988) since Klopproge *et al.* (1989) demonstrated that neutrophils enhance eosinophil LTC_4 synthesis. The effect of indomethacin in our study suggests that the cyclo-oxygenase inhibitor enhanced PAF- and C5a-stimulated LTC_4 synthesis by inhibition of the generation of eosinophil-derived PGE_2, thus reversing the suppression of LTC_4 synthesis by endogenous PGE_2. There is some evidence in support of this hypothesis: firstly, PAF (0.1 μM) induced PGE_2 synthesis in eosinophils to a mean of 23 pg PGE_2 per 10^6 cells corresponding to a concentration of 0.3 nM in the assay system. This PGE_2 production was abolished by 10 μM indomethacin. Secondly, readdition of PGE_2 in the presence of indomethacin potently suppressed PAF-induced LTC_4 synthesis, and at 0.3 nM PGE_2 eosinophil LTC_4 synthesis was substantially inhibited. Our results are therefore in agreement with Kuehl *et al.* (1987) who noted that eosinophil LTC_4 generation was enhanced by indomethacin and inhibited by PGE_2. These authors suggested a similar mechanism to explain the indomethacin-induced increase of neutrophil LTB_4 synthesis by inhibition of neutrophil derived PGE_2 (Kuehl *et al.*, 1984). There is, in addition, some indirect evidence favouring the hypothesis that eosinophil-derived PGE_2 might substantially suppress LTC_4 synthesis. Firstly, the protein kinase A-inhibitor Rp-8-Br-cyclic AMPS augmented PAF-induced eosinophil LTC_4 synthesis to a similar extent as indomethacin (Figure 3). This indicates that in the absence of indomethacin LTC_4 synthesis is suppressed by (endogenous) protein kinase A-activating agents e.g. PGE_2. Secondly, Kok *et al.* (1989) demonstrated that the addition of exogenous arachidonic acid to eosinophils resulted in an increase in PAF-stimulated LTC_4 synthesis. This finding suggests that PAF-induced PGE_2 may inhibit arachidonic acid mobilization and, therefore, LTC_4 synthesis is increased by the addition of exogenous arachidonic acid. A possible alternative explanation for the indomethacin-induced increase in eosinophil LTC_4 generation would be a 'shunting' of arachidonic acid from the cyclo-oxygenase to the lipoxygenase pathway (Stevenson, 1987). This hypothesis, however, has been questioned in view of the findings that leukotriene and prostanoid generation are derived from distinct pools of arachidonic acid (Kuehl *et al.* (1984). Another argument against a mechanism involving 'shunting' of arachidonic acid may be derived from our data demonstrating that a protein kinase A inhibitor augmented LTC_4 synthesis to a similar extent as indomethacin (Figure 3).

The effects of indomethacin on eosinophil-derived PGE_2 and LTC_4 synthesis as described here have been suggested to contribute to the pathogenesis of aspirin-sensitive asthma (ASA) (Kuehl *et al.*, 1987). This is also supported by recent clinical data from Sala *et al.* (1995) showing that inhaled PGE_2 reduced urinary LTE_4 excretion in patients with ASA.

Finally, the question as to whether the presence of indomethacin affects the potency of theophylline and rolipram in suppressing eosinophil LTC_4 generation should be addressed. In view of data indicating that PDE inhibitors simultaneously inhibit eosinophil LTC_4 synthesis (this study) and PGE_2 generation (Shute *et al.*, 1995b) IC_{50} values for PDE inhibition in the absence of indomethacin are hard to predict. We could not perform a complete analysis of the inhibition of LTC_4 synthesis by PDE inhibitors in the absence of indomethacin since eosinophils from only three subjects generated substantial LTC_4 following stimulation with PAF in the absence of indomethacin. In these preliminary experiments, however, an IC_{50} of 30 μM was calculated for the inhibition of PAF-induced LTC_4 generation in the absence of indomethacin by theophylline. This IC_{50} value is not substantially different from those obtained in the presence of indomethacin.

In conclusion, we have demonstrated that the PDE inhibitors rolipram and theophylline concentration-dependently suppress C5a- and PAF-stimulated eosinophil LTC_4 synthesis and eosinophil chemotaxis. No differences were found between eosinophils from normal and atopic subjects with respect to PDE activity and the potency of PDE inhibitors to suppress eosinophil PDE activity, LTC_4 synthesis and chemotaxis. Furthermore, it was found that indomethacin enhanced C5a- and PAF-stimulated LTC_4 synthesis. Most probably, this effect of indomethacin is related to the generation of endogenous PGE_2 which inhibits LTC_4 synthesis in the absence of indomethacin.

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