British Journal of Pharmacology (1996) 119, 1255-1261

The effect of selective phosphodiesterase 3 and 4 isoenzyme inhibitors and established anti-asthma drugs on inflammatory cell activation

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1 This study aimed to evaluate the effects of phosphodiesterase (PDE) inhibitors and currently prescribed anti-asthma drugs for their ability to inhibit inflammatory cell activation *in vitro*.

2 Alveolar macrophages and eosinophils were isolated from the bronchoalveolar lavage (BAL) fluid of ovalbumin (Ovalb)-sensitized guinea-pigs. Opsonized zymosan (OZ) and PAF stimulated leukotriene B_4 (LTB_4) release from eosinophils was measured by radioimmunoassay. Ovalb-induced superoxide generation was measured by reduction of cytochrome C.

3 Monocytes were separated from human peripheral venous blood and mast cells were dispersed from human lung fragments. Lipopolysaccharide (LPS)-induced tumour necrosis factor-α (TNF-α) release from monocytes was measured by ELISA and anti-IgE stimulated histamine release from mast cells was measured by a radioenzymatic method.

4 The β_2 agonist, salbutamol inhibited TNF-α release from monocytes and histamine release from mast cells whilst having no effect on eosinophil-derived LTB_4 release or macrophage superoxide generation.

5 The PDE 3 inhibitor, milrinone produced a concentration-related inhibition of TNF-α release from monocytes which achieved statistical significance at 10^{-5} M but inhibited LTB_4 release from eosinophils and superoxide generation from macrophages only at the highest concentration (10^{-3} M) examined. Milrinone had no effect on histamine release from mast cells.

6 The selective PDE 4 inhibitors, denbufylline and rolipram and the corticosteroid, beclomethasone produced a concentration-related inhibition of LTB_4 release from eosinophils, TNF-α release from monocytes and superoxide generation from alveolar macrophages whilst having no effect on histamine release from mast cells.

7 The mixed PDE 3/4 inhibitor, benzafentrine produced a concentration-related inhibition of LTB_4 release from eosinophils, TNF-α release from monocytes, superoxide generation from alveolar macrophages and histamine release from mast cells.

8 In conclusion these data clearly show that both established anti-asthma medication as well as PDE inhibitors have the potential to inhibit inflammatory cell activation *in vitro* but that the anti-secretory actions of β_2 agonists, corticosteroids and PDE inhibitors are distinct.

Keywords: Phosphodiesterase; eosinophil; monocyte; mast cell; macrophage

Introduction

A number of different cell types including eosinophils, T-lymphocytes, macrophages and mast cells are considered to play a key role in the pathogenesis of bronchial asthma (Durham & Kay, 1985; Lai & Holgate, 1988; Azzawi *et al.*, 1990). These cell types have the potential to release a wide array of mediators including histamine, leukotriene B_4 (LTB_4), tumour necrosis factor-α (TNF-α) and superoxide anions which are thought to contribute to the inflammation and tissue injury observed in the airways of asthmatic patients (reviewed by Barnes *et al.*, 1988).

One means by which this inflammation could be resolved is to either prevent or reduce the activation status of inflammatory cells. Although the precise mechanisms underlying cell activation are not clearly understood, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) is widely accepted to exert a broad suppressant effect on the activation of most inflammatory cells (Torphy & Undem, 1991; Giembycz & Dent, 1992). Agents which elevate cyclic AMP levels thus have the potential to inhibit cellular activation.

The phosphodiesterase (PDE) enzyme catalyses the breakdown of cyclic AMP and guanosine 3′:5′-cyclic monophosphate (cyclic GMP). To date, 7 different isoenzymes of PDE have been characterized on the basis of characteristics such as substrate specificity and their susceptibility to selective inhibitors (Giembycz & Kelly, 1994). The type 4 PDE isoenzyme is the predominant cyclic AMP hydrolysing isoenzyme which has been characterized in inflammatory cells (Torphy & Undem, 1991) and thus inhibition of this isoenzyme might be expected to reduce inflammatory cell activation.

In this study the effects of established anti-asthma drugs in comparison with isoenzyme selective PDE 4 and PDE 3 inhibitors have been examined for their ability to affect inflammatory cell activation.

Methods

Purification of PDE 4 from human neutrophils

The purification of PDE 4 was carried out according to Nielson *et al.* (1990). Polymorphonuclear cells (PMNs) were isolated from heparin (10 u ml^{-1})-treated venous blood drawn from normal donors. Ficoll centrifugation followed by sedimentation with 6% dextran for 1 h at 4°C was used and contaminating erythrocytes were eliminated by lysis. PMNs were then washed once with Tris-HCl (10 mM, pH 7.8) containing $MgCl_2$ (5 mM), EGTA (4 mM) and β-mercaptoethanol (5 mM) prior to being resuspended at a concentration of

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2×10^8 cells ml^{-1} in the same buffer supplemented with leupeptin (1 μM), pepstatin A (1 μM), phenyl-methyl-sulphonyl-fluoride (PMSF) (100 μM) and Triton X-100 (1%). The cell suspension was then homogenized with a Dounce homogenizator (5 times, 10 strokes/time) and centrifuged at 2500 g for 30 min at 4°C in a Sorvall RC-5B centrifuge (rotor SS34).

The supernatant was then applied to a 40×2 cm DEAE-Sepharose CL 6B column previously washed and equilibrated with the same buffer as above but in the absence of Triton X-100. After the sample was completely in the gel, the top of the column was filled with buffer, free of Triton X-100, and the column washed with buffer (250 ml) at a flow rate of 1.3 ml min^{-1}. Isoenzymes of PDE were eluted from the column with a linear 0 to 1 M sodium acetate gradient (solutions were prepared in Triton X-100-free buffer) and 7.5 ml fractions collected. Each fraction was assayed for cyclic AMP metabolizing PDE with 1 μM [^{3}H]-cyclic AMP as the substrate. Fractions containing PDE activity were pooled, dialysed overnight against distilled water and concentrated. Ethylene glycol (30% vol/vol) was added and the samples were stored at −20°C. Under these storage conditions there was no decrease in activity for one month.

Purification of PDE 3 from human platelets

The purification of PDE 3 from human platelets was carried out according to the method used by Simpson *et al.* (1988). Human platelets were purified from a platelet rich plasma (PRP) that was centrifuged at 2200 r.p.m. for 15 min at 4°C. The pellet was made free of contaminating erythrocytes by resuspending it in 5 ml of saline and 15 ml of lysis buffer. The lysis buffer contained in mM: NH_4Cl 155, $KHCO_3$ 10, EDTA 0.1 and was at pH 7.4. After a 15 min period on ice, saline was added to make a final volume of 50 ml and the platelets were centrifuged at 1400 r.p.m. for 10 min at 4°C. The pure platelets were then resuspended in 10 ml of NaCl (145 mM), KCl (5 mM), $MgSO_4$ (1 mM), glucose (10 mM), HEPES (10 mM) and hirudin (0.05 u ml^{-1}) (from stock solution in saline) and could be stored at −30°C for several days. Before the chromatographic step, platelets were thawed and 25 ml of *bis*-Tris (20 mM), β-mercaptoethanol (5 mM), benzamide (2 mM), EDTA (2 mM) and sodium acetate (50 mM) at a pH of 6.5 were added. After the addition of PMSF (50 μM) the platelet suspension was homogenized with a Dounce homogenizator (5 times, 10 strokes/time) and then centrifuged at 2500 g for 20 min at 4°C in a Sorvall RC-5B centrifuge (rotor SS34).

The supernatant was applied to a 17×1.5 cm DEAE-Sepharose CL 6B column previously washed and equilibrated with the same buffer as above. After the sample was completely in the gel, the top of the column was filled with buffer and the column washed with 150 ml of buffer at a flow rate of 1.3 ml min^{-1}. Isoenzymes of PDE were eluted from the column with a linear 50 mM to 1 M sodium acetate gradient (solutions were prepared in buffer) and 7.5 ml fractions collected. Each fraction was assayed for cyclic AMP and cyclic GMP metabolizing PDE with 1 μM ^{3}H-cyclic nucleotides as substrate. Three peaks of activity were obtained. Fractions containing similar PDE activities were pooled, dialysed overnight against distilled water and concentrated. Ethylene glycol (30% vol/vol) was added and the samples were stored at −20°C. Under these storage conditions there was no decrease in activity for one month. The third peak was characterized as PDE 3 by determining the values of K_m for cyclic AMP and cyclic GMP hydrolysis (0.5 μM and 0.2 μM respectively), the negative modulation by cyclic GMP and the potency of selective different PDE inhibitors.

Assay of PDE activity

The PDE activity was assayed by a 'two-step' radioisotopic procedure previously described by Bauer & Schwabe (1980): in the first step the tritiated cyclic nucleotide was hydrolysed to the corresponding 5'-monophosphate; in the second step the 5' monophosphate was further hydrolysed to the corresponding nucleoside with a snake venom nucleotidase (Crotalux atrox).

The incubation buffer (200 μl) consisted of Tris-HCl (40 mM) and $MgCl_2$ 5 mM at pH 8.0 cyclic AMP or cyclic GMP (1 μM) was then added in a volume of 50 μl; [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP were present at about 70,000–80,000 c.p.m. in a volume of 10 μl; the enzyme was present so that the hydrolysis of the substrate was between 10 and 20%. The reaction was started by the addition of the enzyme, carried out for 30 min at 30°C and stopped by warming the samples at 95°C for 3 min and then cooling them on ice; 50 μl of a 1 mg ml^{-1} solution of snake venom nucleotidase was then added and the reaction carried on for another minute at 30°C. The reaction was stopped by application of the samples to QAE A25 Sephadex columns (about 0.8 ml of resin) previously equilibrated with 3 ml of ammonium formate 30 mM, pH 6. The effluent from the columns was immediately collected in scintillation vials; after addition of 2×2 ml of ammonium formate (30 mM) at pH 6 to each column, the eluates were also collected in the same scintillation vials and counted in 15 ml of UltimaGold cocktail (Packard), with a Packard liquid scintillation spectrometer (TRI-CAR 4530) at an efficiency of 60%.

Sensitization of guinea-pigs

Male and female guinea pigs (250–350 g) were supplied by Rodentia (Torre Pallavicina, BG, Italy) and maintained in the animal care unit for at least five days prior to experimentation. On day 1, guinea-pigs were injected with 0.5 ml (5 mg) of ovalbumin (Ovalb) subcutaneously (s.c.) and intraperitoneally (i.p.); the i.p. injection was repeated on day 4.

LTB_4 production by guinea-pig eosinophils

Three weeks after sensitization, airway eosinophilia was induced in guinea-pigs by exposure to an aerosol of Ovalb (1%) for 6–10 s. The following day Ovalb challenge was repeated and 24 h later BAL performed using 5×5 aliquots of saline at 37°C. After elimination of other cells from BAL fluid by a routine adherence method, the purity of eosinophils was about 75–80% (May Gruenwald-Giemsa stain) and the recovery determined by a Coulter Counter (Coulter Corporation, U.S.A.) was about 10^7 eosinophils per guinea-pig.

The activation of the lipoxygenase pathway of eosinophils was performed according to the method of Bruijnzeel & Verhangen (1989): 10^6 eosinophils (100 μl) in a final volume of 0.25 ml of PBS-Dulbecco, were incubated with platelet activating factor (PAF) (1 μM) and drug 10 min (180 min for the steroid) prior to the addition of opsonized zymosan (OZ) (5 mg ml^{-1}). Fifty minutes later the incubation was stopped by the addition of 0.25 ml of cold LTB_4 assay buffer (New England Nuclear). Samples were centrifuged at 1200 r.p.m. for 10 min at 4°C and the content of LTB_4 in the supernatant determined by RIA.

Superoxide production by guinea-pig alveolar macrophages

BAL was performed without previous aerosol challenge and using saline supplemented with EDTA (0.1%). The number of alveolar macrophages was determined in a Coulter Counter (Coulter Corporation, U.S.A.) and a recovery of about 30×10^6/animal was observed. Cell viability was determined by trypan blue exclusion and was >95% with a purity of about 85% (May Gruenwald-Giemsa stain). Alveolar macrophages present in BAL were not further purified by adhesion in order to avoid cell activation. BAL fluid was centrifuged at 1200 r.p.m. for 10 min at 4°C and alveolar macrophages resuspended in Hank's Balanced Salt Solution (HBSS) at a concentration of 4.3×10^6 cells ml^{-1}.

The *in vitro* activation of alveolar macrophages was induced by the specific antigen, Ovalb, and evaluated by cytochrome C reduction as described previously (Brunelleschi *et al.*, 1992). In

a final volume of 0.5 ml of HBSS, 1.5×10^6 alveolar macrophages (0.35 ml) were pre-incubated for 10 min (180 min for the steroid) with the drug and cytochrome C (0.8 mg ml^{-1}). Respiratory burst was induced by Ovalb (1 mg ml^{-1}). The reaction was stopped 60 min later by the addition of 0.75 ml of cold Tris-HCl (50 mM, pH 7.4). After centrifugation at 1400 r.p.m. at 4°C for 5 min, spectrophotometric determination of reduced cytochrome C in the supernatants was performed at 550 nm, using a molar extinction coefficient of 15.5×10^{-3} M^{-1} cm^{-1}. The specificity of cytochrome C reduction was demonstrated by the inhibitory effect of superoxide dismutase (100 u ml^{-1}), a selective scavenger of superoxide anions. The addition of Ovalb determined a time-dependent reduction of cytochrome C (about 5–6 nmol per 10^6 cells after 60 min of incubation), completely prevented by a simultaneous addition of superoxide dismutase 100 u ml^{-1}.

The production of tumour necrosis factor-α by human monocytes

The production of TNF-α by human monocytes was carried out according to the methods described by Linden (1992), Hartmand *et al.* (1993) and Molnar-Kimbar *et al.* (1993). Peripheral venous blood obtained from normal donors was anticoagulated with heparin (10 u ml^{-1}) and mononuclear cells were separated by a modification of the Ficoll-Hypaque technique of Boyum (1968) prior to being suspended in RPMI 1640 plus foetal calf serum (FCS), 10% penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). After determination of total cells with a Coulter counter, the cell suspension was diluted to 2.5×10^6 cells ml^{-1} and incubated in 24 well culture plates (2 ml per well) for 2 h at 37°C in a humid atmosphere of 95% air and 5% CO_2. The mononuclear cell layer was then washed with warm PBS to remove non-adherent cells, such as lymphocytes. Adherent cells were incubated in 2 ml of RPMI 1640 (plus FCS and antibiotics as described above) for 20 h at 37°C in a 5% CO_2 atmosphere. Cells were incubated in the presence or absence of lipopolysaccharide (LPS) from *E.coli* (20 μg ml^{-1} final concentration). Drugs were added (200 μl) at different concentrations prior to stimulation; the test was carried out in triplicate. Supernatants were then transferred to Eppendorf tubes and frozen at −80°C until TNF-α content was determined by ELISA (Amersham). Samples were diluted 1 : 10 with H_2O before assay.

Determination of histamine release from human lung

Histamine release and determination were performed in human lung fragments according to the method described by Verbug *et al.* (1985). Human lung fragments were obtained from surgical resections and mast cells mechanically dispersed by cutting the lung fragments in small pieces and washing them extensively with Tyrode buffer pH 7.4. Each sample contained about 50 mg of tissue in 0.5 ml Tyrode buffer (Composition g^{-1}: NaCl 8.0, $NaHCO_3$ 1.0, KCl 0.1, NaH_2PO_4 0.05, glucose 1.0 plus $CaCl_2$ 0.2 and $MgCl_2$ 0.1). Histamine release was induced by a goat anti-human IgE (10 mg ml^{-1}) and the test carried out at 37°C for 30 min. About 20% of total histamine present in pulmonary mast cells was released. Drugs were pre-incubated with pulmonary tissue 10 min before anti-IgE challenge. Histamine content of the samples was determined by a radioenzymatic method, according to Verburg *et al.* (1985) after diluting the samples 1 : 10 with distilled water. Ultima Gold cocktail (10 ml) was added to each sample and radioactivity counted in a Packard liquid scintillation spectrometer (TRI-CARB 4530) at an efficiency of 60%. The amount of histamine in each sample was determined from a standard curve (obtained by linear regression analysis of fixed amounts of histamine versus the corresponding values of c.p.m.).

Calculations

Data are expressed as mean ± s.e.mean of three experiments carried out in duplicate or triplicate for the various compounds at each concentration tested. Drug-treated cells were compared with vehicle-treated cells by Dunnett's test. The concentration of drug inhibiting the release of the different mediators by 50% (IC_{50}) was calculated by non-linear regression analysis (Graph PAD, ISI Software, U.S.A.).

Materials

Ovalbumin and histamine were obtained from Fluka Chemie AG (Switzerland) and dissolved in NaCl 0.9% solution. Trypan blue, cytochrome C, EDTA, zymosan, platelet activating factor (PAF), leupeptin, pepstatin A, phenyl-methyl-sulphonyl-fluoride (PMSF), hirudin, benzamidine, lipopolysaccharide from *E.coli* (serotype 0127:B8) and nucleotidase (*Crotalux atrox* snake venom) were obtained from Sigma (Milan, Italy). RPMI 1640, Hank's Balanced Salt Solution, FCS, Ficoll hypaque and penicillin streptomycin solution were purchased from Seromed (Milan, Italy). Giemsa's and May-Gruenwald's solutions were from Merck, Germany. Superoxide dismutase was obtained from Boehringer Mannheim Italia (Milan, Italy). LTB_4 RIA kit, histamine-N-methyl-transferase (HNMT), S-adenosyl-L-[^{3}H-methyl]-methionine, [^{3}H]-cyclic AMP and [^{3}H]-cyclic GMP were from New England Nuclear Research (Cologno Monzese, Italy). DEAE-Sepharose CL-6B was obtained from Bio-Rad. Goat anti-human IgE was purchased from Pierce (Rockford, IL). Ultima Gold scintillation cocktail was from Packard Instruments (Pero, Italy).

Drugs

Beclomethasone dipropionate, disodium cromoglycate, salbutamol and theophylline were from Sigma Milan, Italy. RO 20-1724 was from Research Biochemical Incorporate (RBI, Natick, MA, U.S.A.). Rolipram, tibenelast, denbufylline, milrinone, trequinsin, nitraquazone, RP 73401, benzafentrine, zaprinast and B9004-070 were synthesized by the department of Medicinal Chemistry, Zambon Group S.p.A., Bresso, Italy.

Results

Human neutrophil PDE activity

Only one peak of activity was obtained. The enzymatic activity obtained was characterized as PDE 4 by determining the value of K_m for cyclicAMP and cyclicGMP hydrolysis (2 μM and no hydrolysis up to 15 μM respectively) and the potency of different PDE inhibitors.

Human platelet PDE activity

Three peaks of activity were obtained. Two peaks of activity were superimposed using either 1 μM cyclic AMP or cyclicGMP. The first peak, at the same substrate concentration, hydrolyzed much more cyclic GMP than cyclic AMP, according to the characteristics of PDE 5. The third peak, on the contrary, produced similar hydrolysis of the two cyclic nucleotides as determined by K_m values for cyclic AMP or cyclic GMP hydrolysis (0.5 μM and 0.2 μM respectively), the negative modulation by cyclic GMP and the potency of selective different PDE inhibitors and was characterized as PDE 3.

The second peak probably contains both PDE 1 and PDE 2; however, a further characterization (effect of calmodulin, determination of the K_m for the enzyme, inhibition by selective PDE inhibitors) has been performed only for the third peak of PDE activity.

LTB_4 production by guinea-pig eosinophils

PAF (1 μM) alone was unable to stimulate LTB_4 release. OZ (5 mg ml^{-1}) produced a small stimulation of LTB_4 release (200 pg ml^{-1}). However, when eosinophils were stimulated

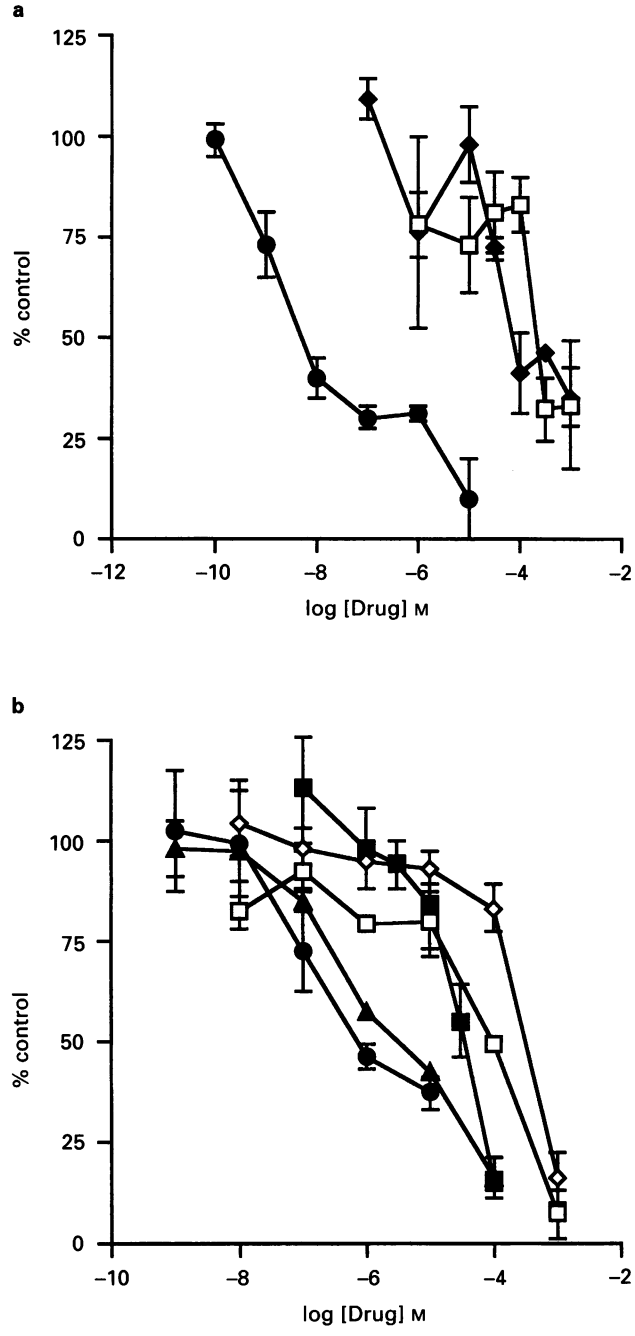

Figure 1 The effect of (a) established anti-asthma drugs: salbutamol (□), theophylline (◆), beclomethasone (●) and (b) PDE inhibitors: benzafentrine (□), denbufylline (●), milrinone (◇), rolipram (▲) and tibenelast (■) on PAF (1 μM) and OZ (5 mg ml^{-1}) stimulated LTB_4 production from guinea-pig eosinophils.

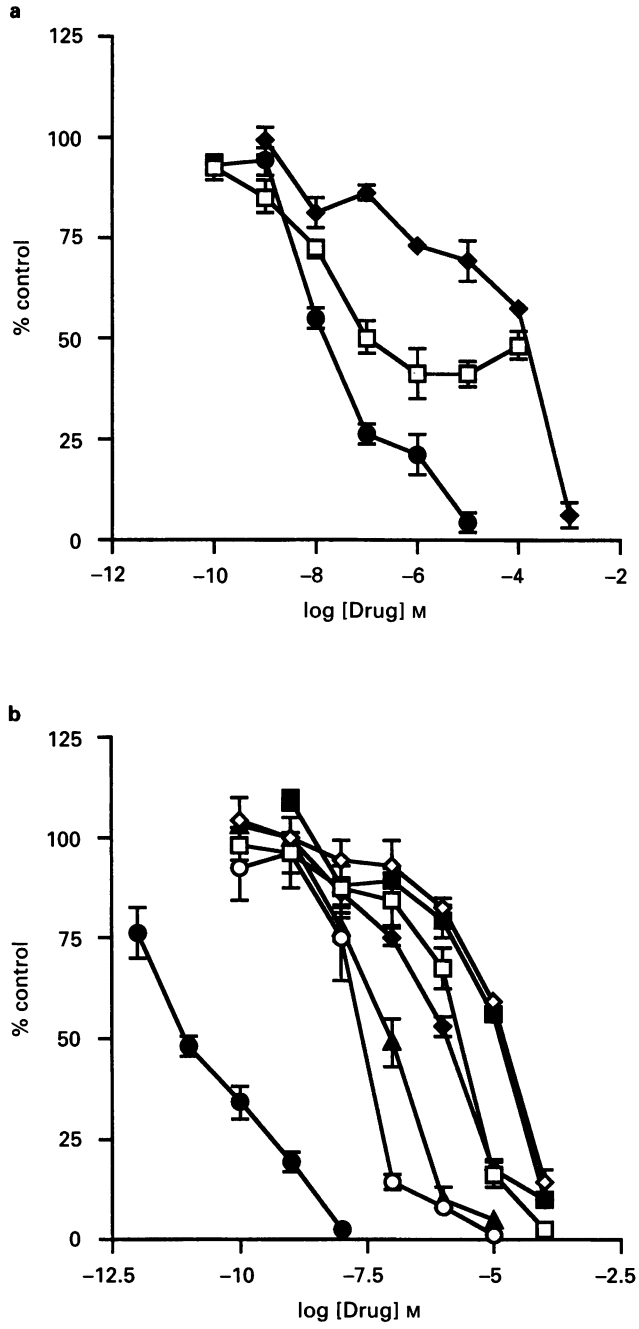

Figure 2 The effect of (a) established anti-asthma drugs: salbutamol (□), theophylline (◆), beclomethasone (●) and (b) PDE inhibitors: benzafentrine (□), denbufylline (○), milrinone (◇), rolipram (▲), tibenelast (■), RO 20-1724 (◆) and RP 73401 (●) on LPS (20 μg ml^{-1}) stimulated TNF-α release from human peripheral blood monocytes.

with OZ (5 mg ml^{-1}) and PAF (1 μM), a large stimulation of LTB_4 release (1411 $\pm$ 70 pg per 10^6 cells) was observed compared with basal LTB_4 release which was 146 $\pm$ 11 pg per 10^6 cells.

Salbutamol, theophylline and beclomethasone all produced a concentration-related inhibition of LTB_4 release from guinea-pig eosinophils which achieved statistical significance at 3.3×10^{-3} M, 10^{-4} M and 10^{-8} M respectively (Figure 1a).

The PDE 4 inhibitors, rolipram, denbufylline and tibenelast, also produced a concentration-related inhibition of LTB_4 release which attained statistical significance at 10^{-6} M, 10^{-5} M and 10^{-4} M respectively. The PDE 3 inhibitor, milrinone was relatively ineffective and significantly inhibited LTB_4 release only at the highest concentration examined, 10^{-3} M. The mixed type 3/4 inhibitor, benzafentrine, produced a concentration-related inhibition of LTB_4 release which achieved statistical significance at 10^{-6} M (Figure 1b). The IC_{50} values for each drug are shown in Table 1.

TNF-α release from human monocytes

LPS (20 μg ml^{-1}) produced a significant stimulation of TNF-α release from human monocytes: 4332 $\pm$ 130 pg ml^{-1} compared with spontaneous basal release of 379 $\pm$ 45 pg ml^{-1}.

Salbutamol, theophylline and beclomethasone produced a

concentration-related inhibition of TNF-α release from monocytes which achieved statistical significance at 10^{-8} M, 10^{-6} M and 10^{-8} M respectively (Figure 2a).

The type 4 inhibitors rolipram, denbufylline, RO 20-1724, RP 73401 and tibenelast, all produced a concentration-related inhibition of TNF-α release from monocytes which achieved statistical significance at 10^{-8} M, 10^{-7} M, 10^{-12} M and 10^{-6} M, respectively. The type 3 PDE inhibitor, milrinone and the mixed type 3/4 inhibitor, benzafentrine also produced a concentration-related inhibition of TNF-α release which achieved statistical significance at 10^{-5} M and 10^{-6} M respectively (Figure 2b). The IC_{50} values for each drug are shown in Table 1.

Superoxide generation by guinea-pig alveolar macrophages

Ovalbumin (1 mg ml^{-1}) produced a significant generation of superoxide production 7.49 ± 0.20 nmol per 10^6 cells compared with basal levels of 1.55 ± 0.11 nmol per 10^6 cells.

Salbutamol and theophylline significantly inhibited superoxide production only at a concentration of 10^{-3} M whereas beclomethasone produced a concentration-related inhibition of superoxide generation which achieved statistical significance at 10^{-7} M (Figure 3a).

The type 4 PDE inhibitors, rolipram, denbufylline and tibenelast produced a concentration-related inhibition of

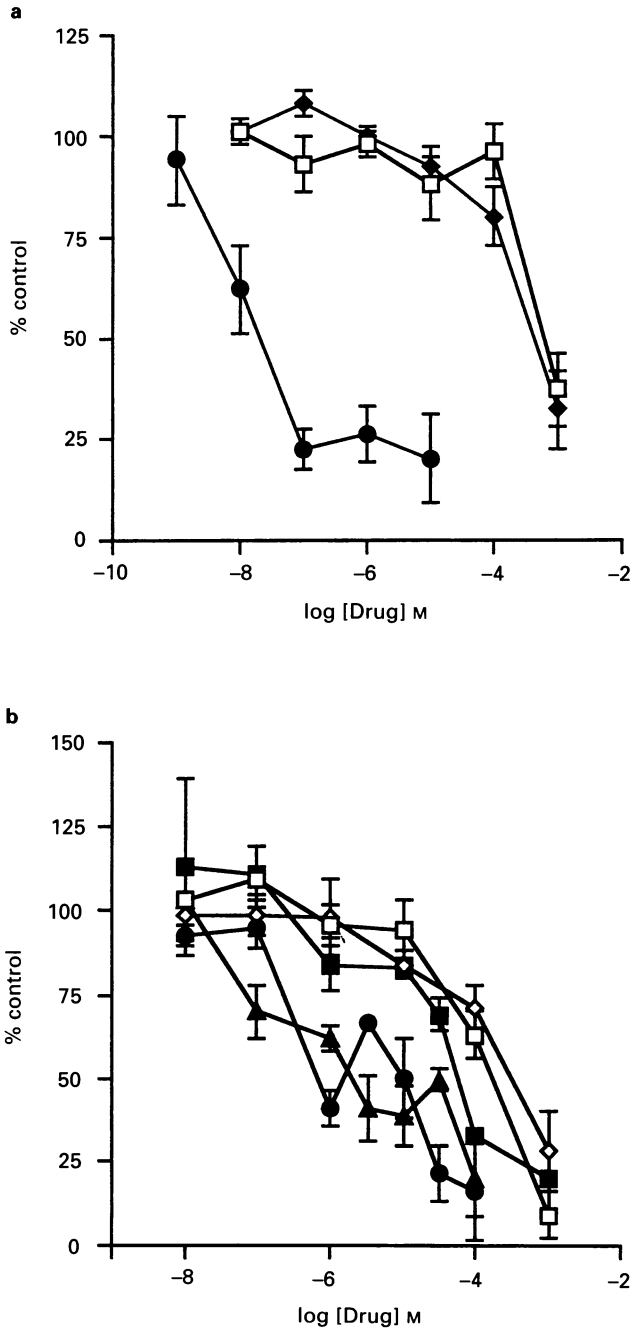

Figure 3 The effect of (a) established anti-asthma drugs: salbutamol (□), theophylline (◆), beclomethasone (●) and (b) PDE inhibitors: benzafentrine (□), denbufylline (●), milrinone (◇), rolipram (▲) and tibenelast (■) on ovalbumin (1 mg ml^{-1}) induced superoxide generation from guinea-pig alveolar macrophages.

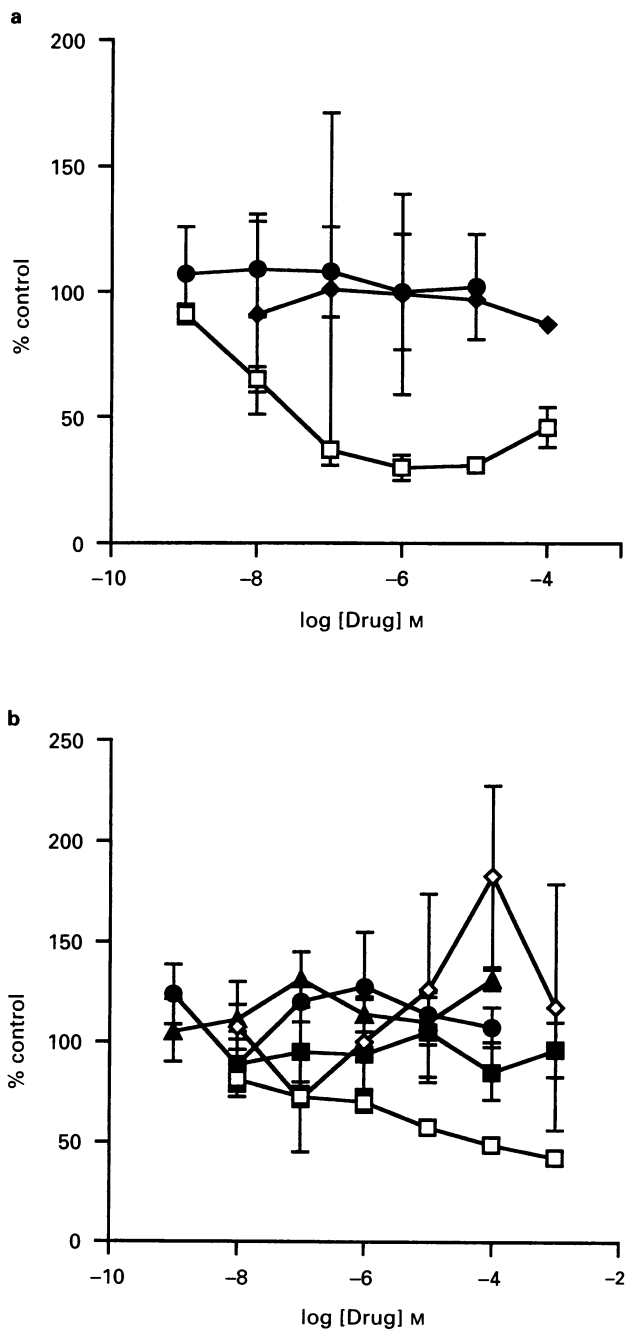

Figure 4 The effect of (a) established anti-asthma drugs: salbutamol (□), theophylline (◆), beclomethasone (●) and (b) PDE inhibitors: benzafentrine (□), denbufylline (●), milrinone (◇), rolipram (▲) and tibenelast (■) on anti-IgE (10 mg ml^{-1}) stimulated histamine release from human lung mast cells.

Table 1 The effect of established anti-asthma drugs and phosphodiesterase inhibitors (IC_{50} values) on mediator release from inflammatory cells

	IC_{50} values (M)			
Drug	*LTB_4*	*O_2^-*	*Histamine*	*TNF-α*
Salbutamol	2.7×10^{-4}	7.0×10^{-4}	1.1×10^{-7}	1.2×10^{-6}
Theophylline	1.8×10^{-4}	5.1×10^{-4}	$>1\times10^{-4}$	4.2×10^{-5}
Beclomethasone	1.1×10^{-8}	1.0×10^{-8}	$>1\times10^{-4}$	6.7×10^{-9}
Benzafentrine	1.0×10^{-4}	1.4×10^{-4}	7.2×10^{-5}	2.3×10^{-6}
Milrinone	3.7×10^{-4}	4.1×10^{-4}	$>1\times10^{-4}$	1.2×10^{-5}
Rolipram	2.7×10^{-6}	4.3×10^{-6}	$>1\times10^{-4}$	7.3×10^{-8}
RO 20-1724				9.0×10^{-7}
RP 73401				1.0×10^{-11}
Tibenelast	3.0×10^{-5}	5.7×10^{-5}	$>1\times10^{-4}$	8.6×10^{-6}
Denbufylline	8.3×10^{-7}	4.4×10^{-6}	$>1\times10^{-4}$	5.6×10^{-8}

superoxide generation which attained statistical significance at 10^{-6} M and 3.3×10^{-5} M respectively. The type 3 inhibitor, milrinone and the mixed type 3/4 inhibitor, benzafentrine inhibited superoxide generation only at 10^{-3} M and 10^{-4} M respectively (Figure 3b). The IC_{50} values for each drug are shown in Table 1.

Histamine release from human lung mast cells

Anti-IgE (10 mg ml^{-1}) produced a significant stimulation of histamine release from human lung mast cells: 425 ng ml^{-1} compared with basal levels of 58 ± 6 ng ml^{-1}.

Salbutamol produced a concentration-related inhibition of histamine release which attained statistical significance at a concentration of 10^{-7} M. In contrast beclomethasone and theophylline were unable to inhibit histamine release from mast cells (Figure 4a).

Similarly the type 4 inhibitors, rolipram, denbufylline and tibenelast and the type 3 inhibitor, milrinone were unable to affect histamine release. However, the mixed type 3/4 inhibitor, benzafentrine produced a concentration-related inhibition of histamine release which achieved statistical significance at 10^{-7} M (Figure 4b). The IC_{50} values for each drug are shown in Table 1.

Discussion

The activation status of inflammatory cells, measured by the release of pro-inflammatory mediators, can be suppressed by established anti-asthma drugs such as corticosteroids and β_2 agonists as well as by PDE inhibitors (Linden, 1992; Hatzelmann *et al.*, 1995). However, the different drug types seem to exert differential effects on different cell types (Linden, 1992). Whilst the precise intracellular mechanism by which these agents inhibit mediator release from inflammatory cells such as eosinophils, macrophages, monocytes, mast cells and lymphocytes is not clear, an increase in cyclic AMP levels is widely associated with an inhibition of the activation status of a number of different inflammatory cell types and this is considered to be the mechanism by which β_2 agonists and PDE inhibitors suppress inflammatory cell activation (Torphy & Undem, 1991). However, there is recent evidence to suggest that β_2 agonists may be exerting their smooth muscle relaxant effects via a cyclic AMP-independent mechanism involving direct coupling of the β_2 receptor to calcium-dependent potassium channels (Miura *et al.*, 1992; Huang *et al.*, 1993).

Our results show that the type 4 PDE inhibitors were effective at inhibiting LTB_4 release from eosinophils whilst the type 3 inhibitor was ineffective, suggesting that the type 4 PDE isoenzyme is involved in the control of LTB_4 release. The type 4 PDE isoenzyme is the only PDE isoenzyme which has been identified in guinea-pig peritoneal eosinophils (Souness *et al.*, 1991). In support of our data the type 4 PDE inhibitors, rolipram, RO 20-1724 and RP 73401 inhibited the respiratory burst in both activated guinea-pig peritoneal eosinophils (Dent *et al.*, 1989; 1991; Souness *et al.*, 1991; 1995; Barnette *et al.*, 1995). Similarly in the present study the corticosteroid, beclomethasone was effective in suppressing LTB_4 generation whilst the β_2 agonist was unable to inhibit LTB_4 release despite the fact that the β_2 receptor has been shown to exist on both human and guinea-pig eosinophils (Yukawa *et al.*, 1990). In support of our findings, Yukawa *et al.* (1990) were unable to demonstrate an inhibitory effect of β_2 agonists on superoxide generation from guinea-pig peritoneal eosinophils.

In the present study, both the type 3 PDE inhibitor and the type 4 PDE inhibitor were effective in inhibiting TNF-α release from human peripheral blood monocytes. Our results would therefore appear to confirm that both the type 3 and type 4 PDE isoenzymes are involved in the control of TNF-α release and that inhibition of either isoenzyme can inhibit TNF-α release. Human peripheral blood monocytes contain almost exclusively the type 4 isoenzyme with a minor PDE 3 activity which accounts for about 15–25% of the total cyclic AMP hydrolysis (Elliot & Leonhard, 1989; Tenor *et al.*, 1995; Verghese *et al.*, 1995). Rolipram and zardaverine were almost equipotent in inhibiting TNF release from mouse peritoneal macrophages (Schade & Schudt, 1993). In our study, beclomethasone and salbutamol were effective in inhibiting TNF release from monocytes.

In the present study the type 4 PDE inhibitors suppressed superoxide generation from guinea-pig alveolar macrophages whilst the type 3 PDE isoenzyme inhibitor was unable to affect superoxide generation. In a separate study, PDE 4 inhibitors reduced superoxide generation from guinea-pig macrophages (Turner & Guermy, 1991). In our study, beclomethasone inhibited superoxide generation from macrophages whereas salbutamol was ineffective. Binding studies suggest that a small population of β_2 receptors exist on human alveolar macrophages (Liggett, 1989). There is evidence to suggest that activation of these receptors causes an elevation in cyclic AMP levels (Liggett, 1989); however, it has been suggested that the presence of contaminating cells may be responsible for this observation (Fuller *et al.*, 1988). Moreover isoprenaline and salbutamol were unable to inhibit zymosan or IgE-induced release of mediators or superoxide anions from human alveolar macrophages (Fuller *et al.*, 1988; Calhoun *et al.*, 1991).

We have also shown that the type 3 PDE inhibitor and the type 4 PDE inhibitors were unable to inhibit anti-IgE stimulated histamine release from mast cells. At present little is known with regard to the PDE profile of human lung mast cells. In one study, PDE 3 and 4 inhibitors were able to reduce histamine release from human lung mast cells (Anderson & Peachell, 1994); however, very high concentrations (300 μM) of PDE inhibitors were used in this study. Like PDE inhibitors, beclomethasone was unable to inhibit histamine release from mast cells in the present study. This is consistent with the fact that steroids have little effect on the early asthmatic response in which mast cell derived histamine is considered to be important in contributing to the acute bronchoconstriction. In contrast to the results with PDE inhibitors and beclomethasone, salbutamol was effective in suppressing mast cell-derived histamine release. This is consistent with the findings of both Peters *et al.* (1982) and Church & Hiroi (1987) who demonstrated that β_2 agonists could inhibit histamine release from human dispersed mast cells, an effect mediated by β_2 receptors present on mast cells in human lung (Butchers *et al.*, 1980).

In conclusion these data clearly show that both established anti-asthma drugs as well as PDE inhibitors have the potential to inhibit inflammatory cell activation *in vitro* but that the antisecretory actions of β_2 agonists, corticosteroids and PDE inhibitors are distinct.

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(Received April 22, 1996
Revised August 14, 1996
Accepted August 27, 1996)