Inhibition of eosinophil cyclic nucleotide PDE activity and opsonised zymosan-stimulated respiratory burst by 'type IV'-selective PDE inhibitors

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1 The cyclic nucleotide phosphodiesterase (PDE) of guinea-pig eosinophils was partially characterized and the effects of selective inhibitors of PDE isoenzymes upon opsonized zymosan (OZ)-stimulated respiratory burst were studied.

2 PDE activity in eosinophil lysates appeared to be membrane-associated, displayed substrate specificity for adenosine 3':5' cyclic monophosphate (cyclic AMP) versus guanosine 3':5' cyclic monophosphate (cyclic GMP) and was insensitive to cyclic GMP or Ca^{2+} and calmodulin.

3 The non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine caused a concentration-dependent inhibition of both OZ-stimulated hydrogen peroxide (H_2O_2) generation and cyclic AMP hydrolysis. The type IV-selective PDE inhibitors, rolipram and denbufylline, also inhibited H_2O_2 generation and cyclic AMP hydrolysis in a concentration-dependent manner whilst SK&F 94120 and Org 9935 (type III-selective) and zaprinast (type Ia or V-selective) were ineffective.

4 Dibutyryl cyclic AMP, a cell-permeable, non-hydrolysable analogue of cyclic AMP, caused a concentration-dependent inhibition of H_2O_2 generation stimulated by OZ. Dibutyryl cyclic GMP was ineffective.

5 It is concluded that eosinophil respiratory burst activity induced by OZ can be regulated by intracellular cyclic AMP and that the levels of cyclic AMP are controlled exclusively by a rolipram- and denbufylline-sensitive PDE isoenzyme that resembles a type IV species.

Keywords: Eosinophils; phosphodiesterases; phosphodiesterase inhibitors; hydrogen peroxide; respiratory burst

Introduction

The second messenger adenosine 3':5' cyclic monophosphate (cyclic AMP) is synthesized from adenosine 5'-triphosphate (ATP) through the activation of adenylyl cyclase by a variety of receptor agonists. Cyclic AMP is known to inhibit the activation of many cell types, including mast cells and basophils (MacGlashan *et al.*, 1983; Undem *et al.*, 1990), lymphocytes and monocytes (Thompson *et al.*, 1976; Elliott & Leonard, 1989), macrophages (Bonta *et al.*, 1984) and neutrophils (Busse & Sosman, 1984; Hoult *et al.*, 1986; Grady & Thomas, 1986). In view of this inhibitory action upon cells involved in inflammatory responses, it has been suggested that agents capable of elevating cyclic AMP levels in such cells may have potential as anti-inflammatory drugs (Persson *et al.*, 1988; Torphy, 1989).

Intracellular levels of cyclic AMP and guanosine 3':5' cyclic monophosphate (cyclic GMP) are regulated by the concerted action of adenylyl and guanylyl cyclase, which catalyse their respective formation, and of 3'-phosphodiesterase (PDE) enzymes which hydrolyse the 3'-ribose phosphate bond to produce the biologically inert 5'-monophosphates. It is now apparent that this latter reaction can be catalysed by a large number of distinct isoenzymes, forming at least five PDE 'families' which may be distinguished by several criteria, including their substrate specificity, kinetic characteristics, tissue distribution, subcellular localization, physico-chemical properties and sensitivity to endogenous cofactors and inhibitors (see Beavo, 1988; Beavo & Reifsnnyder, 1990). While non-selective inhibitors of PDEs, such as theophylline and 3-isobutyl-1-methylxanthine (IBMX) have been available for some time,

potent selective inhibitors have only more recently been identified for certain PDE isoenzymes. For example, zaprinast inhibits the PDE isoenzymes which specifically hydrolyse cyclic GMP, known as type I (Reeves *et al.*, 1987), type Ia (Torphy & Cieslinski, 1990) or type V (Beavo & Reifsnnyder, 1990) PDE (Prigent *et al.*, 1988); SK&F 94120, milrinone, cilostamide and Org 9935, among others, inhibit the low K_m , cyclic GMP-inhibited, cyclic AMP-specific (type III) PDE (Gristwood *et al.*, 1986; Harrison *et al.*, 1986; Shahid *et al.*, 1990) and the cyclic GMP-insensitive low K_m , cyclic AMP-specific (type IV) PDE is selectively inhibited by Ro 20,1724, rolipram and denbufylline (Reeves *et al.*, 1987; Nicholson *et al.*, 1989).

It is likely that eosinophils contribute to the pathogenesis of allergic inflammatory diseases, including bronchial asthma, through the release of a range of cytotoxic basic proteins and oxygen-derived free radicals (Venge, 1981; Gleich *et al.*, 1984). We have demonstrated previously that generation of superoxide anions by guinea-pig and human eosinophils can be inhibited by high concentrations of theophylline (Yukawa *et al.*, 1989) and it has been suggested that xanthines, such as theophylline, may be beneficial in asthma due to their anti-inflammatory, as much as to any bronchodilator, action (Kuehl *et al.*, 1987; Persson *et al.*, 1988). Since theophylline may act by inhibiting cyclic nucleotide hydrolysis (Polson *et al.*, 1978), we have investigated the effects of a cyclic AMP analogue and of non-selective and isoenzyme-selective PDE inhibitors upon opsonised zymosan (OZ)-stimulated respiratory burst in guinea-pig eosinophils. In addition, we have characterized the PDE enzyme activity in these cells in order to determine whether cyclic AMP can inhibit eosinophil function and which PDE isoenzyme is responsible for regulation of intracellular cyclic AMP levels in this cell.

Preliminary accounts of some of these data have been presented to the British Pharmacological Society (Dent *et al.*, 1990; Giembycz *et al.*, 1991).

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Methods

Eosinophil isolation

Eosinophils were harvested by peritoneal lavage of anaesthetized (ketamine 25 mg kg⁻¹, xylazine 5 mg kg⁻¹, i.m.) male Dunkin-Hartley guinea-pigs (700–1300 g body weight) rendered eosinophilic by weekly intraperitoneal injection of normal human serum (1 ml per animal). Lavage cells were washed twice with Hanks balanced salts solution (HBSS) and separated by centrifugation on discontinuous Percoll density gradients, as described by Gärtner (1980). Eosinophil-rich fractions were pooled, washed with HBSS and finally resuspended in the appropriate buffer solution.

Measurement of respiratory burst activity

Respiratory burst activity was measured as the rate of production of hydrogen peroxide (H₂O₂) in the presence of a saturating concentration of superoxide dismutase (SOD). H₂O₂ was assayed by the horseradish peroxidase (HRPO)-catalysed oxidation of scopoletin, quantified by its loss of fluorescence, as described by Root *et al.* (1975). Eosinophils were suspended at a density of 10⁷ cells ml⁻¹ in HEPES-buffered Ca²⁺/Mg²⁺-free Krebs-Ringer bicarbonate solution (KRB, composition, mM: NaCl 138, KCl 6, NaH₂PO₄ 1, NaHCO₃ 5, glucose 5.5 and HEPES 10, pH 7.4) and stored on ice until required; cells prepared in this way remained responsive for up to 7 h. Cells (10⁶ in 100 µl) were added to 875 µl KRB containing MgSO₄ 1 mM, CaCl₂ 1 mM, SOD 30 u, HRPO 1 u and scopoletin 4 µM in polystyrene fluorimeter cuvettes and incubated, with or without drugs, for 2 min at 37°C prior to the addition of 25 µl of zymosan suspension, containing 500 µg of OZ. Kinetic measurements were made at 37°C with a recording spectrofluorophotometer (model RF-5000; Shimadzu Corporation, Kyoto, Japan) at an excitation wavelength of 350 nm and emission wavelength of 460 nm with band widths set to 3 nm. Fluorescence was monitored continuously for 15 min. Rates of reduction of fluorescence were obtained from 1st derivative plots and converted to rates of H₂O₂ generation by comparison with a standard curve constructed for known H₂O₂ concentrations.

Measurement of PDE activity

PDE activity was measured in crude eosinophil lysates by assaying the cleavage of [³H]-cyclic AMP or [³H]-cyclic GMP to its corresponding radiolabelled nucleoside 5'-monophosphate. Eosinophils (10⁸ ml⁻¹) were suspended in lysis buffer, consisting of triethanolamine (TEA) 20 mM, EDTA 1 mM, bacitracin 100 µg ml⁻¹, benzamidine 2 mM, leupeptin 50 µM, phenylmethylsulphonyl fluoride (PMSF) 50 µM and soybean trypsin inhibitor (STI) 10 µg ml⁻¹ (pH 8.0), and lysed by rapid freezing in liquid nitrogen and slow thawing at room temperature followed by ten repeated extrusions through a 23G needle and two rounds of sonication (10 s at 26 µM amplitude); Triton X-100 was added to the resulting homogenate at a final concentration of 0.5% (v/v). It is acknowledged that eosinophils are difficult cells to disrupt (Spry, 1988) and these exceptionally harsh conditions were required to cause lysis of a substantial proportion of the cells. Effective cell disruption was confirmed by the inability of the cell fragments in the resulting suspension to exclude Trypan blue dye. When required, the soluble and particulate fractions were separated by centrifugation of the lysate at 45,000 *g* for 30 min. In further experiments, the particulate fraction was resuspended in ice-cold lysis buffer made up to 500 mM with KCl or supplemented with either Triton X-100 (0.1%, 0.5% or 1% v/v) or sodium deoxycholate (2 mM or 5 mM). This suspension was mixed continuously for 60 min at 4°C and then centrifuged at 45,000 *g* for 30 min; the resulting supernatant was de-salted

(Centriprep-30; Amicon – MWCO 30,000) if necessary, prior to assaying for PDE activity.

PDE activity was assayed according to the method of Thompson & Appelman (1971), as modified by Schwartz & Passonneau (1974). Briefly, 30 µl of eosinophil lysate (or the soluble or particulate fraction thereof) was added to 270 µl of reaction medium containing TEA 40 mM (pH 8.0), EGTA 2 mM, dithiothreitol (DTT) 2 mM, magnesium acetate 5 mM, bovine serum albumin (BSA) 500 µg ml⁻¹, cyclic nucleotide monophosphate supplemented with 2.5–3.5 × 10⁵ d.p.m. [³H]-cyclic nucleotide monophosphate, alkaline phosphatase 0.25 u, approximately 5 × 10³ d.p.m. [¹⁴C]-adenosine and the drug under test of the appropriate vehicle. The concentration of cyclic nucleotide monophosphate was 1 µM throughout except for kinetic experiments in which a range of cyclic AMP concentrations from 1 µM to 1 mM was used. In some experiments cyclic AMP hydrolysis was determined in the presence of cyclic GMP (10 µM) or calmodulin (0.25 u plus 10 µM CaCl₂; EGTA was not included in these assays). Reactions were terminated after 30 min by the addition of 1 ml Dowex AG 1-X8, methanol and water (1:2:1). Slurries were mixed thoroughly and left to stand for 10 min at 4°C before centrifugation at 12,000 *g* for 4 min. The radioactivity in 800 µl aliquots of the resulting supernatants (representing ³H-nucleoside, the product of dephosphorylation of ³H-nucleotide monophosphate by alkaline phosphatase) was determined by liquid scintillation counting, upon the addition of 2 ml ACS II scintillant, at a counting efficiency of approximately 60%. Enzyme activity is expressed as the formation of nucleoside 5'-monophosphate from cyclic nucleotide monophosphate in 1 min per 10⁶ cells at 37°C after correction for the recovery (typically 65–85%) of [¹⁴C]-adenosine.

Data analysis

Concentration-response curves were analysed by least-squares non-linear regression with the InPlot curve-fitting programme (GraphPad Software; San Diego, California, U.S.A.). IC₅₀ values (the concentration of drug required to cause 50% inhibition) are expressed as arithmetic mean ± s.e.mean from 3–10 experiments. Where probability levels are reported, they were calculated by Student's *t* test; a probability of less than 5% was designated as significant.

Materials

Percoll was purchased from LKB/Pharmacia (Milton Keynes, Bucks.). Alkaline phosphatase, bacitracin, benzamidine, BSA (fraction V), calmodulin, cyclic AMP, cyclic GMP, DTT, EDTA, EGTA, H₂O₂ (30% w/v), HRPO, IBMX, leupeptin, magnesium acetate, N⁶-O-2-dibutyryl cyclic GMP, PMSF, scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one), SOD (type IV, from bovine erythrocytes), sodium deoxycholate, STI, TEA, theophylline, Triton X-100 and zymosan A were obtained from Sigma Chemical Co. (Poole, Dorset). N⁶-O-2-dibutyryl cyclic AMP was purchased from Aldrich Chemical Co. (Gillingham, Dorset). [2,8-³H]-cyclic AMP, [8-³H]-cyclic GMP and [8-¹⁴C]-adenosine were supplied by Amersham International (Little Chalfont, Bucks.). Dowex AG 1-X8 (200–400 mesh, acetate form) was obtained from Bio-Rad (Hemel Hempstead, Herts.). Zaprinast (2-*o*-propoxyphenyl-8-azapurin-6-one) was a kind gift of Rhône-Poulenc Röer Inc., Dagenham, Essex. Org 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thien-2-yl)-5-methyl-3(2H)-pyridazinone) and Org 30029 (N'-hydroxy-5,6-dimethoxybenzo[b]thiophene-2-carboxamidamido hydrochloride) were donated by Organon Laboratories, Newhouse, Strathclyde. Rolipram (4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone) was a gift from Schering GmbH, Berlin, Germany. SK&F 94120 ((5-acetimidophenyl)pyrazin-(1H)-one) and denbufylline (1,3-di-*n*-butyl-7-(2'-oxopropyl)-xanthine) were donated by SmithKline

Beecham (Welwyn Garden City, Herts.). Zardaverine (6-(4-difluoromethoxy-3-methoxyphenyl)-3[2H]-pyridazinone) was supplied by Byk Gulden Pharmazeutica, Konstanz, Germany. AH 21-132 (($\pm$)-*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4a,10b-hexahydro-8,9-dimethoxy-2-methyl-benzo-[c[1,6] naphthyridine) was obtained from Sandoz AG, Basel, Switzerland. Drugs were made up as stock solutions of 100 mM in ethanol (IBMX, rolipram, denbufylline), 100 mM NaOH (zaprinast, Org 9935, SK&F 94120, theophylline), dimethyl sulphoxide (Org 30029, zardaverine) or distilled water (AH 21-132) and diluted in the appropriate assay buffer. Dibutyryl cyclic nucleotides were dissolved in buffer at a stock concentration of 100 mM. Zymosan was opsonised for 30 min at 37°C in HBSS containing 20% pooled guinea-pig serum, washed and resuspended in KRB.

Results

Inhibition of respiratory burst

Opsonised zymosan at a concentration of $500 \mu\text{g ml}^{-1}$, a concentration inducing a sub-maximal response, caused a delayed, slow and prolonged generation of H_2O_2 by guinea-pig eosinophils (Figure 1b). The mean peak rate of OZ-stimulated H_2O_2 generation was $2.11 \pm 0.18 \text{ nmol min}^{-1}$ ($n = 20$). This response was inhibited by dibutyryl cyclic AMP, a cell-permeable, non-hydrolysable analogue of cyclic AMP (Figure 1c). This inhibition was concentration-dependent, with an IC_{50} of $322 \pm 97.3 \mu\text{M}$ ($n = 3$), and reached a maximum of $78.8 \pm 8.12\%$ (Figure 2). In contrast, dibutyryl cyclic GMP had little effect at concentrations below 10 mM.

The suppression of H_2O_2 generation by PDE inhibitors is shown in Figure 3 and summarised in Table 1: IBMX and the two type IV-selective inhibitors, rolipram and denbufylline, were effective in reducing OZ-stimulated eosinophil respiratory burst activity while the type V-selective inhibitor zaprinast and type III-selective inhibitors SK&F and Org 9935 were ineffective within the range of concentrations at which they are known to be selective. The concentration-response curves for rolipram and denbufylline were very shallow (Hill coefficient (n_H) = 0.398 ± 0.040 and $n_H = 0.260 \pm 0.049$, respectively) whilst IBMX gave a steeper curve ($n_H = 0.827 \pm 0.062$); IBMX appeared to cause an enhancement of

Figure 2 Effect of dibutyryl cyclic AMP (■) and dibutyryl cyclic GMP (□) on guinea-pig peritoneal eosinophil respiratory burst. Conditions were as described in Figure 1. Rates of generation of H_2O_2 were calculated from the rate of loss of fluorescence of scopoletin. Data represent mean (s.e. mean shown by vertical bars) from 3 separate experiments for dibutyryl cyclic AMP and results from 1 experiment for dibutyryl cyclic GMP.

zymosan-stimulated H_2O_2 generation at lower concentrations, although this effect did not attain statistical significance. Extending the pre-incubation period caused a small reduction in the inhibition induced by $10 \mu\text{M}$ denbufylline (Figure 4), suggesting that the shallowness of the concentration-response curve is unlikely to be due to insufficient equilibration time leading to lower than expected intracellular concentrations of the inhibitor. Concentrations of PDE inhibitors higher than $100 \mu\text{M}$ could not be used for respiratory burst measurements owing to the inhibitory actions of the solvents used for the drugs at these levels.

Characterization of PDE activity

Little hydrolysis of either cyclic AMP or cyclic GMP ($1 \mu\text{M}$) was detected in the soluble fraction of eosinophil homogenates

Figure 1 Loss of fluorescence of scopoletin induced by opsonised zymosan (OZ) and its inhibition by a cyclic AMP analogue. Eosinophils (1×10^6) were pre-incubated without (a and b) or with 1 mM dibutyryl cyclic AMP (c) for 2 min at 37°C prior to addition of buffer (a) or $500 \mu\text{g}$ OZ (b and c). Solid lines represent fluorescence (arbitrary units), broken lines are inverted first derivative curves representing rate of decrease of fluorescence. Figure shows original traces from a single experiment.

Figure 3 Effect of phosphodiesterase (PDE) inhibitors on opsonised zymosan-stimulated H_2O_2 generation (■) and total PDE activity (□) of guinea-pig peritoneal eosinophils. Conditions for H_2O_2 measurements were as described in Figure 1; cyclic AMP hydrolysis was measured at a substrate concentration of $1\ \mu\text{M}$ over 30 min at 37°C . Data represent mean (s.e.mean shown by vertical bars) from 3–10 separate experiments. (a) 3-Isobutyl-1-methylxanthine – non-selective PDE inhibitor; (b) zaprinast – type V PDE inhibitor; (c) SK&F 94120 – type III PDE inhibitor; (d) Org 9935 – type III PDE inhibitor; (e) rolipram – type IV inhibitor; (f) denbufylline – type IV PDE inhibitor.

Table 1 Inhibition of eosinophil respiratory burst and cyclic AMP hydrolysis by phosphodiesterase inhibitors

Drug	IC_{50} (μM)	
	vs. respiratory burst	vs. cyclic AMP ¹ hydrolysis
IBMX	36.0 ± 10.4 (<i>n</i> = 5)	11.2 ± 2.16 (<i>n</i> = 4)
SK&F 94120	> 100 (<i>n</i> = 5)	> 100 (<i>n</i> = 4)
Org 9935	> 100 (<i>n</i> = 3)	≈ 100 (<i>n</i> = 4)
Rolipram	0.485 ± 0.231 (<i>n</i> = 5)	0.160 ± 0.044 (<i>n</i> = 9)
Denbufylline	0.862 ± 0.474 (<i>n</i> = 5)	0.102 ± 0.016 (<i>n</i> = 10)
Zaprinast	> 100 (<i>n</i> = 3)	> 100 (<i>n</i> = 4)

Data represent means $\pm$ s.e.mean. ¹ Cyclic AMP at $1\ \mu\text{M}$.
IBMX: 3-isobutyl-1-methylxanthine

Figure 4 Time-course of inhibition of opsonised zymosan-stimulated H_2O_2 generation by denbufylline ($10\ \mu\text{M}$). Conditions were as described in Figure 1 except that the duration of pre-incubation was varied between 30 s and 4 min. Data points represent mean (s.e.mean shown by vertical bars) from 3 experiments.

(Figure 5a). Increasing the number of cells 10 fold to $10^9\ \text{ml}^{-1}$ did not enhance cyclic nucleotide hydrolysis in this fraction. The apparent lack of soluble PDE activity did not appear to be due to proteolysis of the native enzymes occurring during cell lysis since the inclusion of the protease inhibitors bacitracin, benzamidine, leupeptin, PMSF and STI in the lysis buffer did not uncover detectable PDE activity. Similarly, oxidation of the enzyme at reactive sulphhydryl moieties was unlikely to explain the low soluble PDE activity, as evidenced by the lack of a protective effect by DTT. In contrast both cyclic AMP and, to a lesser extent, cyclic GMP were readily hydrolysed by a PDE activity present in the particulate material (Figure 5a). Treatment of the particulate fraction with a high ionic strength buffer solution (KCl 500 mM) failed to release PDE activity into the soluble phase, as did treatment with the non-ionic detergent Triton X-100 (0.1–1% v/v) or the ionic detergent sodium deoxycholate (2–5 mM).

With the crude eosinophil lysate as the source of PDE, cyclic AMP was the preferred substrate. At a concentration of $1\ \mu\text{M}$, cyclic AMP was hydrolysed by eosinophil PDE at a rate of $1.81 \pm 0.19\ \text{pmol min}^{-1}$ per 10^6 cells (*n* = 37). This rate was significantly (approximately 16 fold) greater than that achieved when the same concentration of cyclic GMP was used as substrate ($0.11 \pm 0.01\ \text{pmol min}^{-1}$ per 10^6 cells, *n* = 6) (Figure 5b). At a concentration of $100\ \mu\text{M}$ however, cyclic GMP was substantially hydrolysed (Figure 5b); such a profile of substrate specificity is a characteristic of type IV PDEs (Beavo, 1988). Cyclic AMP hydrolysis was affected by the inclusion of neither cyclic GMP ($10\ \mu\text{M}$) nor calmodulin (25 u) and CaCl_2 ($10\ \mu\text{M}$) (Figure 5c).

Figure 3 shows the effect of PDE inhibitors upon hydrolysis of cyclic AMP by crude eosinophil lysates. The inhibition of PDE activity closely mimicked the effects upon respiratory burst: rolipram and denbufylline were potent inhibitors while IBMX was less potent but gave a steeper concentration-response curve (Table 2); SK&F 94120, Org 9935 and zaprinast were ineffective at concentrations below $10\ \mu\text{M}$. IC_{50} values for inhibition of cyclic AMP hydrolysis by IBMX, rolipram and denbufylline were lower than those for inhibition of OZ-stimulated H_2O_2 generation (Table 1); however, in all cases the maximum inhibition attainable appeared lower for respiratory burst than for PDE activity (Figure 3). Comparison of a range of non-selective and isoenzyme-selective PDE inhibitors (Table 2) showed inhibition of cyclic AMP hydrolysis by eosinophil lysates to be confined to compounds with PDE IV-

Figure 5 Subcellular distribution, substrate specificity and sensitivity to endogenous factors of guinea-pig eosinophil phosphodiesterase (PDE). Cyclic nucleotide hydrolysis was measured over 30 min at 37°C. (a) Rate of hydrolysis of cyclic AMP (open columns) and cyclic GMP (hatched columns, both at 1 μ M) by the particulate (left hand panel) and soluble (right hand panel) fractions of eosinophil lysates prepared from 10⁸ cells ml⁻¹; (b) hydrolysis of cyclic AMP (open columns) and cyclic GMP (hatched columns) at concentrations of 1 μ M (left hand panel) or 100 μ M (right hand panel) by eosinophil lysates; (c) hydrolysis of cyclic AMP under control conditions (open columns) and in the presence of 10 μ M cyclic GMP (hatched column) or 10 μ M CaCl₂ and 25 u calmodulin (solid column). Data points represent mean (s.e.mean shown by vertical bars) from 3–37 experiments.

inhibitory activity while shallow inhibition curves were seen only with the PDE IV-selective drugs.

Kinetic analysis of cyclic AMP hydrolysis by crude eosinophil lysates yielded a curvilinear Hofstee plot (Figure 6), suggesting the presence of either two distinct enzyme activities or a single enzyme with two hydrolytic sites. Resolution of the curves into two components, assuming conformation of both activities to Michaelis-Menten kinetics (Spears *et al.*, 1971), revealed a high-affinity, low capacity activity ($K_m = 7.97 \mu$ M, $V_{max} = 10.0 \text{ pmol min}^{-1} \text{ per } 10^6 \text{ cells}$) and a low-affinity, high-capacity activity ($K_m = 368 \mu$ M, $V_{max} = 27.8 \text{ pmol min}^{-1} \text{ per } 10^6 \text{ cells}$).

Table 2 Effects of non-selective and isoenzyme-selective phosphodiesterase inhibitors on cyclic AMP hydrolysis by eosinophil lysates

Inhibitor	n	Selectivity	$IC_{50} (\mu\text{M})^1$	n_H
Rolipram	10	IV	0.160 ± 0.044	0.444 ± 0.035
Denbufylline	9	IV	0.102 ± 0.016	0.454 ± 0.033
Zardaverine	3	III/IV	1.63 ± 0.38	0.866 ± 0.866
AH 21-132	4	III/IV	2.83 ± 0.54	0.971 ± 0.055
Org 30029	3	III/IV	9.28 ± 1.81	0.774 ± 0.121
IBMX	4	NS ²	11.2 ± 2.16	0.794 ± 0.053
Theophylline	3	NS ²	477 ± 98.9	1.175 ± 0.167
Zaprinast	4	V	225 ± 37.3	0.956 ± 0.088
Org 9935	4	III	$50.1 \pm 3.4\%^3$	
SK&F 94120	4	III	$32.7 \pm 12.1\%^4$	
Imazodan	3	III	$35.5 \pm 8.3\%^4$	

Data represent mean $\pm$ s.e.mean. ¹ Cyclic AMP at 1 μ M.

² Non-selective. ³ % inhibition at 100 μ M (maximum concentration attainable owing to poor aqueous solubility). ⁴ % inhibition at 1 mM.

IBMX: 3-isobutyl-1-methylxanthine.

Discussion

The identification of isoenzymes of cyclic nucleotide phosphodiesterase which are distinct in their cellular and subcellular distribution and in their sensitivity to specific inhibitors may allow such inhibitors to be used to investigate the mechanisms of regulation of cyclic nucleotides within cells as well as to control specific biological processes in discrete cells or tissues (Corda *et al.*, 1990). Certain cell types may express only a single PDE isoenzyme: for instance murine S49 lymphoma cells contain predominantly the cyclic GMP-insensitive, cyclic AMP-specific, low K_m PDE known as the type IV isoenzyme (Brothers *et al.*, 1982), while human monocytes (Thompson *et al.*, 1976; Elliott & Leonard, 1989) and neutrophils (Wright *et al.*, 1990; Nielson *et al.*, 1990) seem to contain this single cyclic AMP PDE isoenzyme exclusively. These observations prompt speculation that selective inhibitors of this family of PDE isoenzymes may be able to suppress immune reactions; the sensitivity of human neutrophil PDE to Ro 20-1724 (Hoult *et al.*, 1986; Grady & Thomas, 1986) also implies a potential anti-inflammatory capacity for selective PDE IV inhibitors, although it has been reported that such drugs have no effect upon human alveolar macrophage activation (Fuller *et al.*, 1988).

Figure 6 Kinetics of hydrolysis of cyclic AMP (1 μ M–1 mM) by guinea-pig eosinophil phosphodiesterase. Enzyme activity was measured in the particulate fraction of an eosinophil homogenate over 30 min at 37°C. Data are presented according to Hofstee (1952) and resolved into two components according to Spears *et al.* (1971). V_0 = initial reaction velocity; $[S]$ = substrate concentration.

In the present study, we have investigated the effects of both non-selective PDE inhibitors and selective inhibitors of the type III, type IV and type V (also known as type I or Ia) PDE isoenzymes upon the opsonised zymosan-stimulated generation of H_2O_2 by guinea-pig peritoneal eosinophils. We have shown this marker of eosinophil activation to be inhibited by non-selective and PDE IV-selective drugs, while the other drugs tested were ineffective within the range of concentrations at which they retain isoenzyme-selectivity. These findings were confirmed by the sensitivity of crude eosinophil PDE activity exclusively to inhibitors of PDE IV. Concentration-response curves for inhibition of cyclic AMP hydrolysis and OZ (500 μg)-induced H_2O_2 generation were almost superimposable. However, IBMX, rolipram and denbufylline all achieved a maximal inhibition of PDE activity which was greater than their respective maximal inhibition of H_2O_2 generation. Since dibutyryl cyclic AMP was capable of inhibiting only up to 80% of stimulated H_2O_2 generation, it is possible that the mechanism of activation of this response is not completely cyclic AMP-sensitive and, consequently, respiratory burst activity cannot be eliminated by total blockade of cyclic AMP hydrolysis by PDE.

It is striking that the inhibition curves for rolipram and denbufylline against both H_2O_2 generation and cyclic AMP hydrolysis have Hill coefficients significantly lower than unity. The most likely explanations for this phenomenon are: (1) an insufficient equilibration time with inhibitor, (2) the presence of two enzymes with different affinities for the inhibitor or (3) the expression of two hydrolytic sites which have different affinities for cyclic AMP. It is unlikely that equilibrium time was insufficient since the shallow curves are seen in the cell-free PDE assay system as well as the cellular activation measurements and thus diffusion time cannot be critical. In addition, extension of the pre-incubation time led to a slight decrease in the inhibition of H_2O_2 generation by denbufylline. Another explanation is that there may be a second PDE isoenzyme present in eosinophils for which rolipram and denbufylline have a lower affinity. (Non-selective and mixed PDE III/PDE IV inhibitors gave steeper inhibition curves which suggests that, if there are two enzymes, these drugs have an equal affinity for both.) The lack of effect of cyclic GMP upon rates of cyclic AMP hydrolysis in eosinophil lysates indicates the absence of either the cyclic GMP-stimulated (type II) or cyclic GMP-inhibited (type III) isoenzymes, while the insensitivity of the eosinophil PDE activity to Ca^{2+} and calmodulin confirms the absence of either of the Ca^{2+} /calmodulin-dependent (type Ib and Ic; Torphy & Cieslinski, 1990) isoenzymes. The great preference for cyclic AMP as substrate, in conjunction with the lack of inhibition by zaprinast, implies that the cyclic GMP-specific (type V) isoenzyme makes no contribution to the PDE activity of guinea-pig eosinophils. Perhaps a more likely interpretation is that two distinct type IV isoenzymes may be present within these cells: all of the inhibition curves for rolipram and denbufylline can be fitted to a two-component model with no significant change in goodness-of-fit, whilst a Hofstee plot of the data from kinetic experiments was clearly curvilinear, suggesting the possible presence of a second enzyme. However, the very high K_m value obtained for the low-affinity cyclic AMP-hydrolytic activity, suggests that this may not represent a genuine PDE enzyme. Our inability to solubilize the particulate PDE isoenzyme(s) present, added to the presence within the cell lysates of highly cationic proteins, has precluded the resolution of separate eosinophil PDE isoenzymes by ion-exchange chromatographic methods.

It is noteworthy that two distinct denbufylline-sensitive PDEs have been isolated from rat gastrocnemius (C.D. Nicholson & R. Wilke, personal communication) and two

cyclic GMP-insensitive, rolipram-inhibited PDEs are present in soluble extracts of rat liver (Lavan *et al.*, 1989) and kidney (Hoey & Houslay, 1990). In addition, a second, high-affinity, membrane-associated binding site for [^3H]-rolipram has been identified in the central nervous systems of several species but its identity as a PDE enzyme has not been confirmed (Schneider *et al.*, 1986); both binding sites identified in the latter study had similar affinities for IBMX, which would be consistent with the steeper inhibition curves obtained with IBMX (compared to those for denbufylline) in the present study.

An enhancement of activity of a plasma membrane cyclic AMP PDE has been demonstrated as a result of phosphorylation by an insulin receptor-activated tyrosyl kinase (Pyne *et al.*, 1989) and it is possible that particular enzymes may exist in multiple states with different activities. It is apparent that the solubilization and purification of the guinea-pig eosinophil PDE must be achieved in order to determine the number and precise nature of the isoenzyme(s) present.

Unlike membrane-bound PDE isoenzymes in other tissues e.g. type II in rat liver plasma membranes (Pyne *et al.*, 1986) and cardiac ventricle (Timouyasse *et al.*, 1989); type III in rat adipocytes (Degerman *et al.*, 1987) and in canine and Rhesus monkey cardiac ventricle (Weishaar *et al.*, 1987); type IV in rat liver plasma membranes (Pyne *et al.*, 1987) which are effectively solubilized by treatment with various ionic and non-ionic detergents, the membrane-associated PDE present in guinea-pig eosinophils was exceedingly resistant to detergent solubilization. Indeed, both Triton X-100 and deoxycholate were completely ineffective at solubilizing the particulate PDE. This suggests that the eosinophil PDE is an integral membrane component.

The IC_{50} for inhibition of guinea-pig peritoneal eosinophil PDE by IBMX obtained in the present study (11.2 μM) is similar to that reported for the type IV PDE isoenzyme obtained from murine S49 lymphoma cells (14 μM), human monocytes (16 μM) and canine trachea (9.1 μM). In contrast, the IC_{50} obtained for rolipram (0.16 μM) is approximately 10 to 20 fold lower than that reported for type IV PDE of human monocytes (2.7 μM) and canine trachea (1.5 μM) (Brothers *et al.*, 1982; Elliott & Leonard, 1989; Galvan & Schudt, 1990), but only 4.5 fold lower than its IC_{50} for PDE IV from human neutrophils (Nelson *et al.*, 1990). This discrepancy appears to suggest that multiple rolipram-sensitive isoenzymes exist and that an enzyme with greater sensitivity to rolipram predominates in neutrophils and eosinophils. The IC_{50} of rolipram for inhibition of formylmethionyl-leucyl-phenylalanine-stimulated respiratory burst in human neutrophils was 0.71 μM , in close accordance with our figure for the OZ-stimulated eosinophil response (0.49 μM). Consistent with our findings in eosinophils, the only cyclic AMP PDE enzyme activity in human neutrophils was confined exclusively to the particulate fraction with characteristics indicative of it being a type IV isoenzyme. Furthermore, analysis of cyclic AMP hydrolysis by this PDE clearly shows curvilinear kinetics (Wright *et al.*, 1990).

In conclusion, we have now identified a membrane-associated type IV PDE as the predominant isoenzyme in guinea-pig peritoneal eosinophils by demonstration of the inhibition of stimulated respiratory burst and PDE activity exclusively by inhibitors of the type IV isoenzyme. Thus, drugs such as rolipram and Ro 20-1724 which have already been identified as bronchodilators both *in vitro* (Torphy *et al.*, 1988) and *in vivo* (Harris *et al.*, 1988) may also act to reduce airway eosinophilia and inflammation.

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References

- BEAVO, J.A. (1988). Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv. Second Messenger Phosphoprot. Res.*, **22**, 1–38.
- BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 150–155.
- BONTA, I.L., ADOLFS, M.J.P. & FIEREN, M.W.J.A. (1984). Cyclic AMP levels and their regulation by prostaglandins in peritoneal macrophages of rats and humans. *Adv. Cyclic Nucleotide Res.*, **17**, 615–620.
- BROTHERS, V.M., WALKER, N. & BOURNE, H.R. (1982). Increased cyclic nucleotide phosphodiesterase activity in a mutant S49 lymphoma cell. Characterization and comparison with wild type enzyme activity. *J. Biol. Chem.*, **257**, 9349–9355.
- BUSSE, W.W. & SOSMAN, J.M. (1984). Isoproterenol inhibition of isolated human neutrophil function. *J. Allergy Clin. Immunol.*, **73**, 404–410.
- CORDA, D., LUINI, A. & GARATTINI, S. (1990). Selectivity of action can be achieved with compounds acting at second messenger targets. *Trends Pharmacol. Sci.*, **11**, 471–473.
- DEGERMAN, E., BELFRAGE, P., NEWMAN, A.H., RICE, K.C. & MANGANILLO, V.C. (1987). Purification of the putative hormone-sensitive cyclic AMP phosphodiesterase from rat adipose tissue using a derivative of cilostamide as a novel affinity ligand. *J. Biol. Chem.*, **262**, 5797–5807.
- DENT, G., GIEMBYCZ, M.A. & BARNES, P.J. (1990). Inhibition of eosinophil oxygen radical production by type IV- but not type III-selective cAMP phosphodiesterase inhibitors. *Br. J. Pharmacol.*, **99**, 163P.
- ELLIOTT, K.R.F. & LEONARD, E.J. (1989). Interactions of formylmethionyl-leucyl-phenylalanine, adenosine, and phosphodiesterase inhibitors in human monocytes. Effects on superoxide release, inositol phosphates and cAMP. *FEBS Letts.*, **254**, 94–98.
- FULLER, R.W., O'MALLEY, G., BAKER, A.J. & MACDERMOT, J. (1988). Human alveolar macrophage activation: inhibition by forskolin but not β -adrenoceptor stimulation or phosphodiesterase inhibition. *Pulmonary Pharmacol.*, **1**, 101–106.
- GALVAN, M. & SCHUDT, C. (1990). Actions of the phosphodiesterase inhibitor zardaverine on guinea-pig ventricular muscle. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **342**, 221–227.
- GÄRTNER, I. (1980). Separation of human eosinophils in density gradients of polyvinylpyrrolidone-coated silica gel (Percoll). *Immunology*, **40**, 133–136.
- GIEMBYCZ, M.A., RABE, K., DENT, G. & BARNES, P.J. (1991). Identification and partial characterisation of cyclic nucleotide-dependent phosphodiesterase activities in guinea-pig peritoneal eosinophils. *Br. J. Pharmacol.*, (Abstract in press.)
- GLEICH, G.J., FRIGAS, E., FILLEY, W.V. & LOEGERING, D.A. (1984). Eosinophils and bronchial inflammation. In *Asthma: Physiology, Immunopharmacology, and Treatment*. ed. Kay, A.B., Austen, K.F. & Lichtenstein, L.M. pp. 195–210. London: Academic Press.
- GRADY, P.J. & THOMAS, L.L. (1986). Characterisation of cyclic nucleotide phosphodiesterase activities in resting and N-formylmethionylleucylphenylalanine-stimulated human neutrophils. *Biochim. Biophys. Acta*, **885**, 282–293.
- GRISTWOOD, R.W., EDEN, R.J., OWEN, D.A.A. & TAYLOR, E.M. (1986). Pharmacological studies with SKF 94120, a novel positive inotropic agent with vasodilator activity. *J. Pharm. Pharmacol.*, **38**, 452–459.
- HARRIS, A.L., CONNELL, M.J., FERGUSON, E.W., WALLACE, A.M., GORDON, R.J., PAGANI, E.D. & SILVER, P.J. (1989). Role of low K_m cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **251**, 199–206.
- HARRISON, S.A., REIFSNYDER, D.H., GALLIS, B., CADD, G.G. & BEAVO, J.A. (1986). Isolation and characterisation of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs. *Mol. Pharmacol.*, **29**, 506–514.
- HOEY, M. & HOUSLAY, M.D. (1990). Identification and selective inhibition of four distinct soluble forms of cyclic nucleotide phosphodiesterase activity from kidney. *Biochem. Pharmacol.*, **40**, 193–202.
- HOFSTEE, B.J.H. (1952). On the evaluation of the constants V_m and K_m in enzymatic reactions. *Science*, **116**, 329–331.
- HOULT, J.R.S., NOURSHARGH, S. & WALDEN, F.M. (1986). Cyclic AMP phosphodiesterase in human neutrophils: identification and stimulation in intact cells. *Br. J. Pharmacol.*, **89**, 81P.
- KUEHL, F.A. JR., ZANETTI, M.E., SODERMAN, D.D., MILLER, D.K. & HAM, E.A. (1987). Cyclic AMP-dependent regulation of lipid mediators in white cells. A unifying concept for explaining the efficacy of theophylline in asthma. *Am. Rev. Respir. Dis.*, **136**, 210–213.
- LAVAN, B.E., LAKEY, T. & HOUSLAY, M.D. (1989). Resolution of soluble cyclic nucleotide phosphodiesterase isoenzymes, from liver and hepatocytes, identifies a novel IBMX-insensitive form. *Biochem. Pharmacol.*, **38**, 4123–4136.
- MACGLASHAN, D.W. JR., SCHLEIMER, R.P., PETERS, S.P., SCHULMAN, E.S., ADAMS, G.K., SOBOTKA, A.K., NEWBALL, H.H. & LICHTENSTEIN, L.M. (1983). Comparative studies of human basophils and mast cells. *Fed. Proc.*, **42**, 2504–2509.
- NICHOLSON, C.D., JACKMAN, S.A. & WILKE, R. (1989). The ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase and its affinity for adenosine receptors and the adenosine re-uptake site. *Br. J. Pharmacol.*, **97**, 889–897.
- NIELSON, C.P., VESTAL, R.E., STURM, R.J. & HEASLIP, R. (1990). Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. *J. Allergy Clin. Immunol.*, **86**, 801–808.
- PERSSON, C.G.A., ERJEFALT, I. & GUSTAFFSON, B. (1988). Xanthines – symptomatic or prophylactic in asthma. *Agents Actions*, Suppl. **23**, 137–156.
- POLSON, J.B., KRZANOWSKI, J.J., GOLDMAN, A.L. & SZENTIVANYI, A. (1978). Inhibition of human pulmonary phosphodiesterase activity by therapeutic levels of theophylline. *Clin. Exp. Pharmacol. Physiol.*, **5**, 535–540.
- PRIGENT, A.F., FOUQUIER, S., NEMOZ, G., ANKER, G., PACHECO, H., LUNGER, C., LEBEC, A. & STOCLET, J.C. (1988). Comparison of cyclic nucleotide phosphodiesterase isoforms from rat heart and bovine aorta. *Biochem. Pharmacol.*, **37**, 3671–3681.
- PYNE, N.J., COOPER, M.E. & HOUSLAY, M.D. (1986). Identification and characterisation of both the cytosolic and particulate forms of cyclic GMP-stimulated, cyclic AMP phosphodiesterase from rat liver. *Biochem. J.*, **234**, 325–334.
- PYNE, N.J., COOPER, M.E. & HOUSLAY, M.D. (1987). The insulin- and glucagon-stimulated “dense-vesicle” high affinity cyclic AMP phosphodiesterase from rat liver: purification, characterisation and inhibitor sensitivity. *Biochem. J.*, **242**, 33–42.
- PYNE, N.J., CUSHLEY, W., NIMMO, H.G. & HOUSLAY, M.D. (1989). Insulin stimulates the tyrosyl phosphorylation and activation of the 52 kDa peripheral plasma-membrane cyclic AMP phosphodiesterase in intact hepatocytes. *Biochem. J.*, **261**, 897–904.
- REEVES, M.L., LEIGH, B.M. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 535–541.
- ROOT, R.K., METCALF, J., OSHINO, N. & CHANCE, B. (1975). H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation and some regulating factors. *J. Clin. Invest.*, **55**, 945–955.
- SCHNEIDER, H.H., SCHMIECHEN, R., BEZINSKI, M. & SEIDLER, J. (1986). Stereospecific binding of the antidepressant rolipram to brain protein structures. *Eur. J. Pharmacol.*, **127**, 105–115.
- SCHWARTZ, J.P. & PASSONEAU, J.V. (1974). Cyclic AMP-mediated induction of cyclic AMP phosphodiesterase of C_6 glioma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3844–3848.
- SHAHID, M., COTTNEY, J.E., WALKER, G.B., MCINDEWAR, I., BRUIN, J.C., SPIERS, I., LOGAN, R.T. & NICHOLSON, C.D. (1990). Pharmacological and biochemical effects of Org 9335: a cardiotonic agent with positive inotropic, Ca-sensitising and vascular relaxant properties. *Br. J. Pharmacol.*, (Abstract in press.)
- SPEARS, G., SNEYD, J.G.T. & LOTEN, E.G. (1971). A method for deriving kinetic constants for two enzymes acting on the same substrate. *Biochem. J.*, **125**, 1149–1151.
- SPRY, C.J.F. (1988). *Eosinophils: a Comprehensive Review and Guide to the Scientific and Medical Literature*. Oxford: Oxford University Press.
- THOMPSON, M.M. & APPELMAN, W.J. (1971). Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry*, **10**, 311–316.
- THOMPSON, W.J., ROSS, C.P., PLEDGER, W.J. & STRADA, S.J. (1976). Cyclic adenosine 3':5'-monophosphate phosphodiesterase: distinct forms in human lymphocytes and monocytes. *J. Biol. Chem.*, **251**, 4922–4929.
- TIMOUYASSE, L., PRIGENT, A.F., NEMOZ, G., LAGARDE, M. & PACHÉCO, H. (1989). Differential susceptibility to biological detergents of the particulate cGMP-stimulated phosphodiesterase from rat heart: preservation of the allosteric properties of the solubilised enzyme. *Biochem. Int.*, **19**, 287–299.
- TORPHY, T.J. (1989). Phosphodiesterase isoenzymes: potential targets for novel anti-asthmatic agents. In *New Drugs for Asthma*. ed. Barnes, P.J. pp. 66–77. London: IBC.
- TORPHY, T.J. & CIESLINSKI, L.B. (1990). Characterization and selec-

- tive inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol. Pharmacol.*, **37**, 206–214.
- TORPHY, T.J., BURMAN, M. & HUANG, L.B.F. (1988). Role of phosphodiesterase (PDE) isoenzymes in regulating cyclic nucleotide content in canine trachealis. *FASEB J.*, **2**, A1066.
- UNDEM, B.J., TORPHY, T.J., GOLDMAN, D. & CHILTON, F.H. (1990). Inhibition by adenosine 3':5'-monophosphate of eicosanoid and platelet-activating factor biosynthesis in the mouse PT-18 mast cell. *J. Biol. Chem.*, **265**, 6750–6758.
- VENGE, P. (1981). The eosinophil granulocyte and its role in the inflammatory process. In *The Inflammatory Process*. ed. Venge, P. pp. 305–327. Stockholm: Almqvist & Wiksell.
- WEISHAAR, R.E., KOBYLARZ-SINGER, P.C., QUADE, M.M., STEFFEN, R.P. & KAPLAN, H.R. (1987). Multiple molecular forms of phosphodiesterase and the regulation of cardiac muscle contractility. *J. Cyclic Nucl. Protein Phosphorylation Res.*, **11**, 513–527.
- WRIGHT, C.D., KUIPERS, P.J., KOBYLARZ-SINGER, D., DEVALL, L.J., KLINKEFUS, B.A. & WEISHAAR, R.E. (1990). Differential inhibition of human neutrophil functions: role of cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase. *Biochem. Pharmacol.*, **40**, 699–707.
- YUKAWA, T., KROEGEL, C., CHANEZ, P., DENT, G., UKENA, D., CHUNG, K.F. & BARNES, P.J. (1989). Effect of theophylline and adenosine on eosinophil function. *Am. Rev. Respir. Dis.*, **140**, 327–333.

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