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Suppression of eosinophil function by RP 73401, a potent and selective inhibitor of cyclic AMP-specific phosphodiesterase: comparison with rolipram

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1 We have investigated the inhibitory potency of RP 73401, a novel, highly selective and potent inhibitor of cyclic AMP-specific phosphodiesterase (PDE IV), against partially-purified PDE isoenzymes from smooth muscle and the particulate PDE IV from guinea-pig eosinophils. The inhibitory effects of RP 73401 on the generation of superoxide ($.O_2^-$), major basic protein (MBP) and eosinophil cationic protein (ECP) from guinea-pig eosinophils have also been studied.

2 RP 73401 potently inhibited partially-purified cyclic AMP-specific phosphodiesterase (PDE IV) from pig aortic smooth muscle (IC_{50} = 1.2 nM); it was similarly potent against the particulate PDE IV from guinea-pig peritoneal eosinophils (IC_{50} = 0.7 nM). It displayed at least a 19000 fold selectivity for PDE IV compared to its potencies against other PDE isoenzymes. Rolipram was approximately 2600 fold less potent than RP 73401 against pig aortic smooth muscle PDE IV (IC_{50} = 3162 nM) and about 250 times less potent against eosinophil PDE IV (IC_{50} = 186 nM).

3 Solubilization of the eosinophil particulate PDE IV increased the potency of rolipram 10 fold but did not markedly affect the potency of RP 73401. A similar (10 fold) increase in the PDE IV inhibitory potency of rolipram, but not RP 73401, was observed when eosinophil membranes were exposed to vanadate/glutathione complex (V/GSH).

4 Reverse transcription polymerase chain reaction (RT-PCR), using primer pairs designed against specific sequences in four distinct rat PDE IV subtype cDNA clones (PDE IV_{A-D}), showed only mRNA for PDE IV_D in guinea-pig eosinophils. PDE IV_D was also the predominant subtype expressed in pig aortic smooth muscle cells.

5 RP 73401 ($K_{i\,app}$ = 0.4 nM) was 4 fold more potent than (±)-rolipram ($K_{i\,app}$ = 1.7 nM) in displacing [^{3}H]-(±)-rolipram from guinea-pig brain membranes.

6 In intact eosinophils, RP 73401 potentiated isoprenaline-induced cyclic AMP accumulation (EC_{50} = 79 nM). RP 73401 also inhibited leukotriene B_4-induced generation of $.O_2^-$ (IC_{50} = 25 nM), and the release of major basic protein (IC_{50} = 115 nM) and eosinophil cationic protein (IC_{50} = 7 nM). Rolipram was 3–14 times less potent than RP 73401.

7 Thus RP 73401 is a very potent and selective PDE IV inhibitor which suppresses eosinophil function suggesting that it may be a useful agent for the treatment of inflammatory diseases such as asthma. The greatly different inhibitory potencies of rolipram against PDE IV from smooth muscle and eosinophils (in contrast to the invariable effects of RP 73401) are unlikely to be attributable to diverse PDE IV subtypes but suggest distinct interactions of the two inhibitors with the enzyme.

Keywords: Cyclic AMP-phosphodiesterase; RP 73401; rolipram; eosinophil; superoxide; major basic protein; eosinophil cationic protein

Introduction

Much attention has recently focused on the therapeutic potential of adenosine 3':5'-cyclic monophosphate (cyclic AMP)-specific phosphodiesterase (PDE IV) inhibitors for the treatment of asthma (Torphy & Undem, 1991; Giembycz, 1992; Raeburn *et al.*, 1993). This stems primarily from the wide-ranging anti-inflammatory properties of PDE IV inhibitors *in vitro* and *in vivo*. For example, the archetypal PDE IV inhibitor, rolipram, suppresses functional responses (e.g. generation of reactive oxygen species, cytokines, mediators and cytotoxic proteins) in mast cells (Torphy *et al.*, 1992a), basophils (Peachell *et al.*, 1992), monocytes (Semmler *et al.*, 1993), macrophages (Schade & Schudt, 1993), neutrophils (Fonteh *et al.*, 1993; Wright *et al.*, 1990), lymphocytes (Epstein *et al.*, 1984; Robicsek *et al.*, 1991) and eosinophils (Dent *et al.*, 1991; Souness *et al.*, 1991). *In vivo*, PDE IV inhibitors suppress microvascular leakage (Raeburn & Karlsson, 1993), eosinophil accumulation (Underwood *et al.*, 1993), passive cutaneous anaphylaxis (Davies & Evans, 1973) and anaphylactic bronchospasm (Underwood *et al.*, 1993). As well as their anti-inflammatory effects, PDE IV inhibitors relax airways smooth muscle and exhibit bronchodilator activity *in vivo* (Harris *et al.*, 1989). Furthermore, PAF-induced bronchial hyperresponsiveness in guinea-pigs is attenuated by rolipram (Raeburn & Lewis, 1991).

Asthma is a disease characterized by variable airways obstruction and bronchial hyperresponsiveness which has been linked to mucosal inflammation and, in particular, the influx and activation of eosinophils (Barnes *et al.*, 1988; Kay, 1985). PDE IV inhibitors, with their dual anti-inflammatory and bronchodilator activities, may be useful for treating both the symptoms and the underlying causes of the disease. We have synthesized a PDE IV inhibitor, RP 73401 (3-cyclopentyloxy-*N*-[3,5-dichloro-4-pyridyl]-4-methoxybenzamide) (Figure 1) (Ashton *et al.*, 1994) and compared its inhibitory activities against PDE IV preparations from smooth muscle

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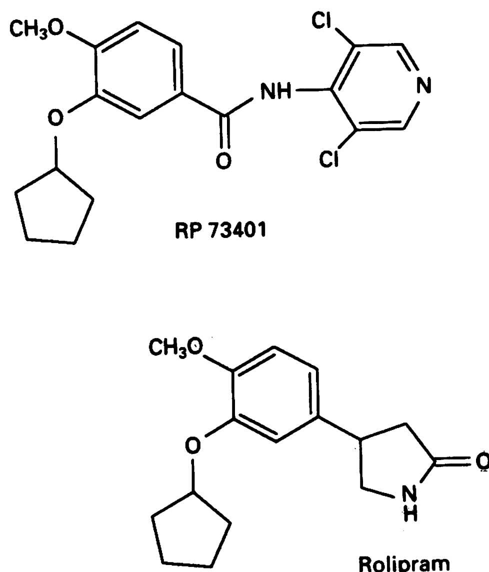

Figure 1 Structures of RP 73401 and rolipram.

and eosinophils with those of the standard PDE IV inhibitor, rolipram. We have also investigated the effects of these compounds on the physiological responses of eosinophils to exogenously applied stimuli.

Methods

Preparation of guinea-pig peritoneal eosinophils

Male, Dunkin-Hartley guinea-pigs (250–400 g) were injected (i.p.) with 0.5 ml of donor horse serum twice weekly for 4 weeks. At least 5 days after the final injection, the guinea-pigs were killed by CO_2 asphyxiation. A ventral incision was made into the peritoneum and 30 ml of Hank's buffered salts solution (HBSS) without Ca^{2+} (Gibco, U.K. Ltd) were poured into the abdominal cavity. The abdomen was gently massaged for approximately 1 min and the peritoneal exudate aspirated and centrifuged (250 *g*, 10 min, 4°C). The supernatant was discarded and the pellet washed once (HBSS, 10 ml) and resuspended in HBSS containing 5% (v/v) foetal calf serum (FCS). Aliquots (1 ml) of the cell suspension were layered onto a discontinuous (55%, 65%, 70%, v/v) Percoll gradient prepared in resuspension buffer. The gradients were centrifuged (250 *g*, 20 min, 20°C) and the normo-dense and hypo-dense eosinophils, which accumulated at the 65%/70% and 55%/65% Percoll gradient interfaces, respectively, were resuspended in HBSS (10 ml). Total cell counts were determined with a Coulter counter (Z1) and differential cell counts were obtained from cytospin slides fixed in methanol and stained with Wright-Giemsa. Cell viability was greater than 95% and eosinophil purity greater than 97%.

Preparation of eosinophil subcellular fractions

Cells ($100-200 \times 10^6$), suspended in HBSS, were centrifuged (250 *g*, 10 min, 4°C), the supernatant removed and the resulting pellet resuspended in 5 ml of homogenization buffer (Tris/HCl, 20 mM [pH 7.5]; $MgCl_2$, 2 mM; dithiothreitol, 1 mM; ethylenediaminetetraacetic acid (EDTA), 5 mM; sucrose, 0.25 M; *p*-tosyl-L-lysine-chloromethylketone (TLCK), 20 μM; leupeptin, 10 $\mu g\ ml^{-1}$; aprotinin, 2000 $u\ ml^{-1}$). Cells were homogenized with a Dounce homogenizer (10 strokes). The homogenate was centrifuged (105,000 *g*, 60 min), the supernatant collected and the pellet resuspended in an equal volume of homogenization buffer.

Solubilization of membrane-associated cyclic AMP PDE from eosinophils

The membrane-bound cyclic AMP PDE was solubilized by homogenizing freshly prepared membranes with a Dounce homogenizer (10 strokes) in homogenization buffer containing deoxycholate (0.5%) and NaCl (100 mM). The homogenate was centrifuged (100,000 *g*, 30 min) and the supernatant containing the solubilized activity removed.

Partial purification of smooth muscle PDE isoenzymes

Ca^{2+}/calmodulin-dependent PDE (PDE I), cyclic GMP-inhibited PDE (PDE III), cyclic AMP-specific PDE (PDE IV) and cyclic GMP-specific PDE (PDE V) were isolated from pig aortic smooth muscle. Isoenzymes were partially purified by DEAE-trisacryl anion exchange chromatography (IBF, Villeneuve La Garenne, France) from the 100,000 *g* supernatant fraction of aortic smooth muscle strips which were prepared and processed as previously described (Souness & Scott, 1993). Cyclic GMP-stimulated PDE (PDE II) was partially purified by the same homogenization and chromatography procedure except that the source of the isoenzyme was fresh bovine cervical trachealis isolated according to Giembycz & Barnes (1991).

Measurement of PDE activity

PDE activity was determined by the two-step radioisotope method of Thompson *et al.* (1979). The reaction mixture contained (mM): Tris-HCl 20 (pH 8.0), $MgCl_2$ 10, 2-mercaptoethanol 4, ethyleneglycol-*bis*-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 0.2, bovine serum albumin 0.05 $mg\ ml^{-1}$. Unless otherwise stated, the substrate concentration was 1 μM.

The IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds were determined from concentration (0.1 nM to 40 μM)-response curves. At least three concentration-response curves were generated for each agent.

For kinetic studies, the concentration of cyclic AMP was varied while the amount of ^{3}H-labelled cyclic AMP remained constant.

Categorisation of PDE isoenzymes

The nomenclature adopted in this paper for the different cyclic nucleotide PDEs is based on that of Beavo & Reifsnyder (1990). PDE IV subtypes referred to in this paper are based on four rat cDNA nucleotide sequences (PDE IV_{A-D}/rpdes 1-4) as reported by Swinnen *et al.* (1989).

Preparation of Na_3VO_4/GSH complex (V/GSH)

The V/GSH complex was prepared according to Souness *et al.* (1985). Briefly, reduced glutathione (GSH – 224 mM) and Na_3VO_4 (112 mM) were mixed together to form a green complex; 10 μl of this solution was added to the 400 μl assay mix, immediately prior to addition of enzyme. The final concentration of GSH and Na_3VO_4 were 2.8 mM and 1.4 mM, respectively.

Measurement of eosinophil cyclic AMP accumulation

For measurement of cyclic AMP, freshly prepared eosinophils (1×10^6 cells ml^{-1}) were preincubated in HBSS containing Ca^{2+} and Mg^{2+}. To test the effects of PDE IV inhibitors, compounds (0.0064 μM – 100 μM) were routinely added to cell suspensions for 10 min, after which incubations were continued for a further 2 min, either in the absence or presence of isoprenaline (10 μM). Incubations were terminated with 50 μl of 100% trichloroacetic acid (TCA).

The TCA extract was briefly sonicated (10 s), centrifuged

(3000 g) for 15 min and the supernatant removed to a clean tube. TCA was removed with 3 washes of water saturated diethyl ether (5 vols). The last traces of ether were removed by gassing with nitrogen, and sodium acetate (pH 6.2) was added to a final concentration of 50 mM. Samples were acetylated and cyclic AMP quantified by radioimmunoassay (RIA, NEN Chemicals GmbH).

Measurement of eosinophil superoxide generation

Superoxide anion ($.O_2^-$) generation was determined as the superoxide dismutase (SOD) inhibitable reduction of *p*-iodonitrotetrazolium violet (INTV) as previously described (Souness *et al.*, 1991). Briefly, cells (1×10^6/well) were incubated for 15 min (37°C) in 96-well microtitre plates in HBSS (0.225 ml) containing INTV (0.5 mg ml^{-1}) with either vehicle control (0.1% dimethyl sulphoxide) or PDE inhibitors (0.001 – 10 μM). SOD (1 mg ml^{-1}) was added to some incubations to determine what proportion of dye reduction was attributable to $.O_2^-$ generation. Following the initial 15 min incubation, leukotriene B_4 (LTB_4) (10 nM) was added and incubations continued for a further 10 min. The cells were then centrifuged (500 g, 10 min) and the supernatant aspirated. The pellet was solubilized by incubation overnight at room temperature in DMSO containing 0.6 M HCl and the absorbance of the reduced dye measured at 492 nm (Titertek Multiskan MCC/340). The results are expressed as % inhibition of the SOD inhibitable $.O_2^-$ release.

Measurement of major basic protein (MBP) and eosinophil cationic protein (ECP)

Cells (1×10^6/ml) were incubated for 15 min (37°C) in 96-well microtitre plates in HBSS (0.225 ml) containing cytochalasin B (5 μg ml^{-1}) with vehicle control (0.1%) or PDE IV inhibitors (0.001 – 1 μM). Leukotriene B_4 (LTB_4, 10 nM) was then added and the incubation was continued for a further 10 min; 200 μl of the supernatant was removed from each well and stored at –20°C prior to quantification of MBP and ECP.

MBP was measured by a modification of the antigen capture ELISA previously described (Hunt *et al.*, 1993). Briefly, microtitre plates (Falcon Pro-Bind) were coated with monoclonal antibodies for MBP (BMK13, Sera-Lab, 3 μg ml^{-1}) in sodium carbonate (15 mM)/sodium bicarbonate (35 mM) coating buffer. After overnight incubation (4°C), the plates were washed with phosphate buffered saline (PBS) containing Tween-20 (0.05%, v/v). The remaining free binding sites were blocked with BSA (1%). Eosinophil supernatants (100 μl undiluted), or eosinophil MBP (0 – 800 μg ml^{-1}, prepared and stabilized as previously described (Gleich *et al.*, 1973; Wassom *et al.*, 1979)), were then added to the plates and incubated for 2 h (with shaking). Plates were washed three times with PBS/Tween-20. Detection antibody (alkaline phosphatase labelled IgG diluted 1:400 in PBS) was added to the wells and incubated for 90 min at room temperature. Following second antibody incubation, the plates were washed three times with PBS/Tween-20 and the detection substrate bromochloroindophenol (BCIP) was added and incubated for 60 min at 37°C. Plates were then read at 590 nm.

ECP was measured by a similar antigen capture ELISA using polyclonal anti-human ECP antibodies (DA178, Kabi Pharmacia Diagnostics, Milton Keynes, U.K.). The assay was identical to that described above for MBP except that primary antibody was coated onto 96-well plates at a concentration of 5 μg ml^{-1}, calibration curves for standard human ECP (Kabi Pharmacia Diagnostics) were in the range 0 – 3.2 μg ml^{-1} and the alkaline phosphatase detection antibody was used at a 1:500 dilution. Results are expressed as % inhibition of LTB_4-induced MBP or ECP release.

Measurement of [³H]-(±)-rolipram binding to brain membranes

(±)-Rolipram was brominated in CCl_4 and dispatched to Amersham International where it was tritiated by catalytic reduction with palladium and charcoal. The specific radioactivity of the [^{3}H]-(±)-rolipram was 24.7 Ci $mmol^{-1}$.

Guinea-pig brain membranes were prepared and the binding assay was performed as described by Schneider *et al.* (1986) with [^{3}H]-rolipram (2 nM) and membrane samples corresponding to 500 μg of brain tissue.

Expression of mRNA for PDE IV subtypes

Total RNA was prepared with a Total RNA Separator kit from Clontech. Briefly, $1 \times 10^5 – 1 \times 10^6$ cells were homogenized in denaturing solution: guanidinium thiocyanate, 4 M; Na^+ citrate, 25 mM [pH 7.0]; sarcosyl, 0.5%; 2-mercaptoethanol, 0.1 M, followed by phenol/chloroform/isoamyl alcohol extraction and isopropanol precipitation according to the manufacturers protocol. First strand cDNA synthesis and cDNA amplification were performed using a GeneAMP RNA PCR kit (Perkin Elmer Cetus). Primers specific for the different rat PDE IV subtypes were designed by Mr I. Giddings (Molecular Medicine Unit, King's College, London) based on reported sequences (Swinnen *et al.*, 1989) from the Genbank data-base (Table 1). PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin Elmer Cetus) set for 35 – 55 cycles. The temperatures set for PCR were: denature 95°C, 30 s; primer anneal 55°C, 120 s; primer extension 72°C, 180 s. Primer fragments were analysed by electrophoresis on 2% agarose gels, and DNA was visualized by ethidium bromide staining. Each set of primers was first tested using rat brain and rat lung poly (A^+) mRNA (Clontech). Agarose gel electrophoresis of ethidium bromide-stained DNA fragments generated after 55 cycles of amplification showed that there

Table 1 Primers used in RT-PCR experiments

Oligonucleotide	*	*Sequences* (position 1-5′)	*Fragment size* (bases)
5 pde 1	5 PDE IV_C	CYYAYGTGGCYTAYCACAACA	535
3 pde 1	3 PDE IV_C	GTCCACTGGCGGTAGAGGGGT	
5 pde 2	5 PDE IV_A	CYYAYGTGGCYTAYCACAACA	174
3 pde 2	3 PDE IV_A	TTAGGAACTGGTTGGAGAGCG	
5 pde 3	5 PDE IV_D	CYYAYGTGGCYTAYCACAACA	331
3 pde 3	3 PDE IV_D	ATGGCCATTTTCCTTAAAGAT	
5 pde 4	5 PDE IV_B	CYYAYGTGGCYTAYCACAACA	593
3 pde 4	3 PDE IV_B	CATTCCCCTCTCCCGTTCTTT	

Y = Inosine.
*Nomenclature of Beavo & Reifsnyder (1990).

was one DNA fragment in each reaction corresponding to the size of the expected PDE IV subtype fragment as defined by the primers used in the PCR. To verify their identity, PCR products were sequenced with Sequenase DNA sequencing kits (Amersham, Bucks, U.K.).

Inhibition of methacholine-induced contraction of guinea-pig trachealis

Guinea-pig trachealis was prepared and organ bath studies performed as described previously (Souness *et al.*, 1994). The relaxant effects of RP 73401 and rolipram were determined in tissue contracted with a concentration of methacholine which produced a 30% of maximal contraction (EC_{30}) as determined by computerised linear regression analysis. The mean EC_{30} value for methacholine was 0.09 ± 0.01 μM ($n = 10$). IC_{50} values (means ± s.e.mean, $n = 6$) were taken as the concentration of relaxant producing 50% relaxation of methacholine-induced contraction.

Materials

RP 73401 and rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone] were synthesized by the department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd (Dagenham, Essex, U.K.). Cyclic [2,8-^{3}H]-AMP (41 Ci $mmol^{-1}$) and cyclic [8-^{3}H]-GMP (13.8 Ci $mmol^{-1}$) were from Amersham International (Amersham, Bucks., U.K.). The cyclic AMP radioimmunoassay kit was purchased from NEN Chemicals GmbH. Cell culture reagents were from Gibco BRL (Paisley, Scotland). Donor horse serum was purchased from Flow Laboratories Ltd. (Irvine, Scotland). The monoclonal antibody for major basic protein (BMK10) was from Sera-Lab (Crawley Down, Sussex, U.K.) and the monoclonal antibody for eosinophil cationic protein (DA178) was from Pharmacia (Milton Keynes, U.K.). The Clontech total RNA Separator kit was purchased from Cambridge BioScience (Cambridge, U.K.) and the GeneAMP RNA PCR kit was from Perkin Elmer Cetus (Vaterstetten, Germany). All other chemicals were obtained from Sigma Chemical Co. or BDH Chemicals (both of Poole, Dorset, U.K.) and Rhône-Poulenc Ltd. (Eccles, Manchester, U.K.). Male Dunkin-Hartley guinea-pigs were purchased from a local supplier.

Results

Inhibition of partially-purified PDEs from pig aortic and bovine tracheal smooth muscle

RP 73401 potently and selectively inhibited PDE IV from pig aortic smooth muscle (Table 2). It was at least 19,000 times less potent aginst PDEs I, II (bovine trachea), III and V. In comparison, rolipram was a relatively weak, though still selective (>70 fold), inhibitor of PDE IV (Table 2). A similar inhibitory potency difference between the two compounds was observed against partially-purified PDE IV from bovine tracheal smooth muscle ($-\log IC_{50}$ [M]: RP 73401 = 9.00 ± 0.06, $n = 4$; $-\log IC_{50}$ [M]: rolipram = 5.71 ± 0.05, $n = 3$).

Inhibition of eosinophil PDE IV by RP 73401 and rolipram – effects of solubilization and V/GSH

RP 73401 displayed similar potencies against the eosinophil and pig aortic PDE IV (Tables 2 and 3). In contrast, and as reported previously (Souness & Scott, 1993), rolipram was over 10 times more potent as an inhibitor of membrane-bound PDE IV from eosinophils than of the partially-purified PDE IV from pig aorta (Table 3). Solubilization with deoxycholate plus NaCl or treatment of membranes with V/GSH increased the potency of rolipram by a further 10 fold (Table 3). Neither solubilization nor V/GSH had any significant effect on the inhibitory potency of RP 73401 (Table 3). The potency of rolipram ($-\log IC_{50}$ [M] = 6.18 ± 0.08, $n = 4$) decreased when tested against membrane-bound PDE IV from eosinophils which had been stored at −80°C; however, the potency of RP 73401 ($-\log IC_{50}$ [M] = 9.00 ± 0.06, $n = 4$) was unaffected by these storage conditions.

The inhibitory effects of RP 73401 and rolipram against the solubilized, eosinophil PDE IV are not competitive (Figure 2). Indeed, the nature of the inhibition is difficult to interpret in view of the marked non-linearity of the Lineweaver-Burk plots, especially in the presence of inhibitor.

Table 2 Inhibition of smooth muscle phosphodiesterases (PDEs) by RP 73401 and rolipram

	– log IC_{50} [M] ± s.e.mean	
PDE type	*RP 73401*	*Rolipram*
I	4.36 ± 0.01 ($n = 5$)	<3 ($n = 4$)
II	4.14 ± 0.02 ($n = 3$)	3.70 ± 0.01 ($n = 3$)
III	3.83 ± 0.26 ($n = 5$)	3.18 ± 0.15 ($n = 4$)
IV	8.93 ± 0.02 ($n = 4$)	5.50 ± 0.08 ($n = 5$)
V	4.66 ± 0.10 ($n = 5$)	3.05 ± 0.10 ($n = 3$)

PDEs I and V were measured with 1 μM cyclic GMP substrate, the former in the presence of 2 mM $CaCl_2$ + 10 u ml^{-1} calmodulin. PDEs III and IV were measured using 1 μM cyclic AMP substrate. PDE II was measured with 1 μM cyclic AMP substrate in the presence of 10 μM cyclic GMP.

Table 3 Effects of solubilization and vanadate/glutathione complex (V/GSH) on the inhibitory potencies of RP 73401 and rolipram against eosinophil PDE IV

	– log IC_{50} [M] ± s.e.mean	
Treatment	*RP 73401*	*Rolipram*
None	9.15 ± 0.20 ($n = 3$)	6.73 ± 0.09 ($n = 8$)
Solubilization	9.07 ± 0.18 ($n = 4$)	7.55 ± 0.09 ($n = 7$)
V/GSH	8.68 ± 0.06 ($n = 4$)	7.94 ± 0.11 ($n = 7$)

Cyclic AMP PDE activity in the bound, particulate (−/+V/GSH) and deoxycholate/NaCl-solubilized preparations was measured with 1 μM substrate.

Stimulation of eosinophil cyclic AMP accumulation

Both RP 73401 and rolipram potently enhanced the accumulation of cyclic AMP in intact eosinophils elicited by isoprenaline (Figure 3). RP 73401 ($-\log EC_{50}$ [M] = 7.11 ± 0.13, $n = 3$) was approximately 4 fold more potent than rolipram ($-\log EC_{50}$ [M] = 6.66 ± 0.02, $n = 3$). Little or no effect of either PDE inhibitor was observed in the absence of isoprenaline (data not shown), nor was an increase in cyclic AMP accumulation observed after a 10 min incubation with isoprenaline alone.

Inhibition of LTB_4-induced release of $.O_2^-$, MBP and ECP from eosinophils

RP 73401 was approximately 6 fold more potent than rolipram in inhibiting LTB_4-stimulated superoxide generation from eosinophils (Figure 4a). The $-\log IC_{50}$ [M] values were 7.60 ± 0.08, ($n = 4$) and 6.73 ± 0.04 ($n = 4$) for RP 73401 and rolipram, respectively. RP 73401 was also a very potent inhibitor of LTB_4-induced ECP ($-\log IC_{50}$ [M]: 8.16 ± 0.10, $n = 4$) generation but was less potent in suppressing MBP ($-\log IC_{50}$ [M]: 6.94 ± 0.04, $n = 4$) release (Figure 4b,c). Rolipram was less potent than RP 73401 against both cationic proteins, exhibiting inhibitory potencies ($-\log IC_{50}$ [M]) of 6.99 ± 0.07, ($n = 4$) and 6.22 ± 0.02 ($n = 4$) on ECP and MBP generation, respectively (Figure 4b,c).

Displacement of rolipram binding from brain membranes

RP 73401 was a very potent inhibitor of [^{3}H]-(±)-rolipram binding to membranes prepared from guinea-pig brains ($-\log\ K_{i\,app}$ [M] = 9.42 ± 0.11, $n = 3$) (Figure 5). (±)-Rolipram was about 4 fold less potent in displacing the tritiated ligand ($-\log\ K_{i\,app}$ [M] = 8.77 ± 0.07, $n = 3$).

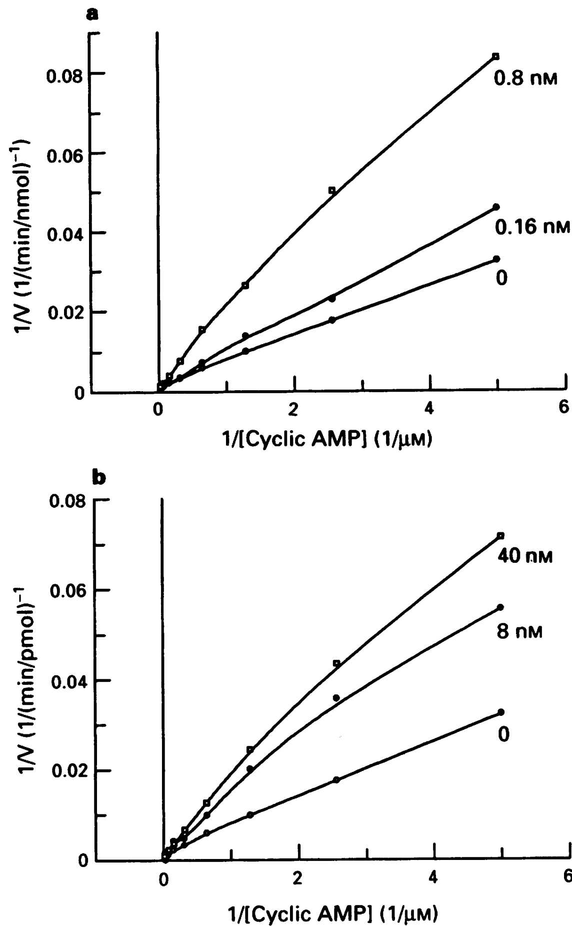

Figure 2 Kinetic analysis of RP 73401 (a) and rolipram (b) inhibition of solubilized eosinophil PDE IV. The data represent Lineweaver-Burk plots in the presence of increasing concentrations of RP 73401 (a) or rolipram (b).

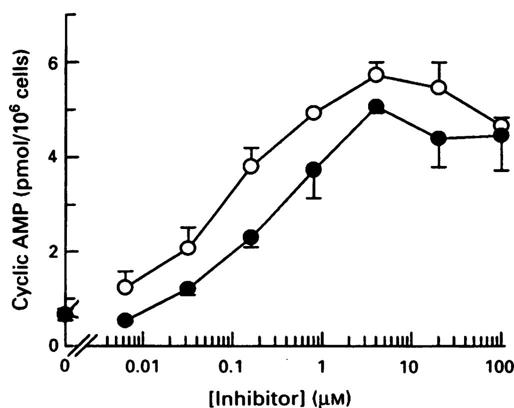

Figure 3 Enhancement of isoprenaline-induced cyclic AMP accumulation in intact eosinophils by RP 73401 and rolipram. Cells were preincubated for 10 min in the presence of the indicated concentrations of RP 73401 (○) and rolipram (●) before exposure to isoprenaline (10 μM) for a further 2 min. Control cells contained 0.91 ± 0.25 pmol of cyclic AMP/10^6 cells ($n = 5$). The results represent the means ± s.e.mean ($n = 3$).

Expression of PDE IV subtypes in eosinophils and smooth muscle

RT-PCR was used to determine the PDE IV subtypes expressed in eosinophils and smooth muscle. Primers designed to recognize sequences in the genes of 4 different rat PDE IV subtypes (PDE IV_{A-D}/rpdes 1–4) (Swinnen *et al.*, 1989) were employed in these studies (Table 1). Each set of primers was first tested employing reverse transcribed rat brain-, guinea-pig brain- and rat lung-poly (A^+) mRNA (Clontech) from which PCR amplified cDNA fragments of the correct size

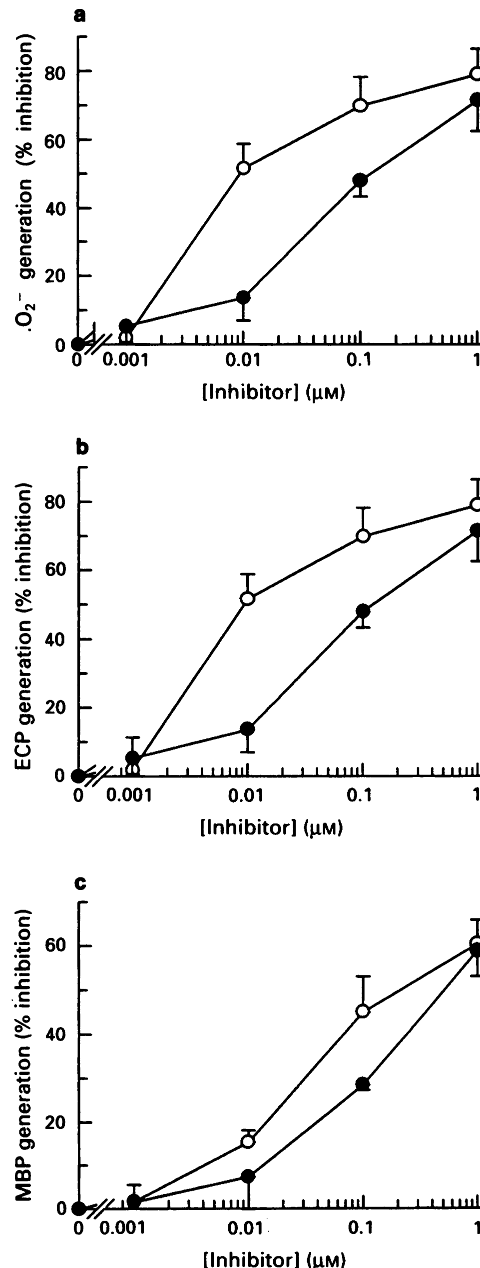

Figure 4 Inhibition of leukotriene B_4 (LTB_4)-induced .O_2^-, major basic protein (MBP) and eosinophil cationic protein (ECP) release from eosinophils by RP 73401 and rolipram. Cells were preincubated with the indicated concentrations of RP 73401 (○) and rolipram (●) for 15 min. LTB_4 (10 nM) was then added and the incubations were continued for a further 10 min before cells were pelleted and the medium was assayed for .O_2^- (a), ECP (b) and MBP (c). Resting cells released 285 ± 42 ng ECP/10^6 cells and 38 ± 6 ng MBP/10^6 cells. After stimulation with LTB_4, media contained 1460 ± 120 ng ECP/10^6 cells and 268 ± 31 ng MBP/10^6 cells. The results, expressed as % inhibition of LTB_4-alone values, represent means ± s.e.mean of 4 separate incubations.

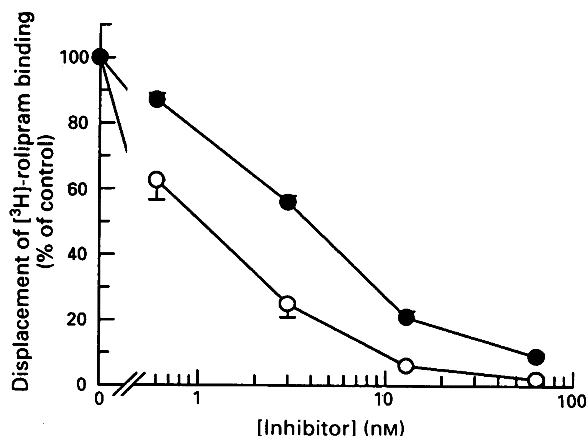

Figure 5 Displacement of [^{3}H]-($\pm$)-rolipram from brain membranes by RP 73401 and rolipram. [^{3}H]-($\pm$)-rolipram binding to brain membranes was measured in the presence of the indicated concentrations of RP 73401 (O) and rolipram (●). The results represent means $\pm$ s.e.means ($n = 3$).

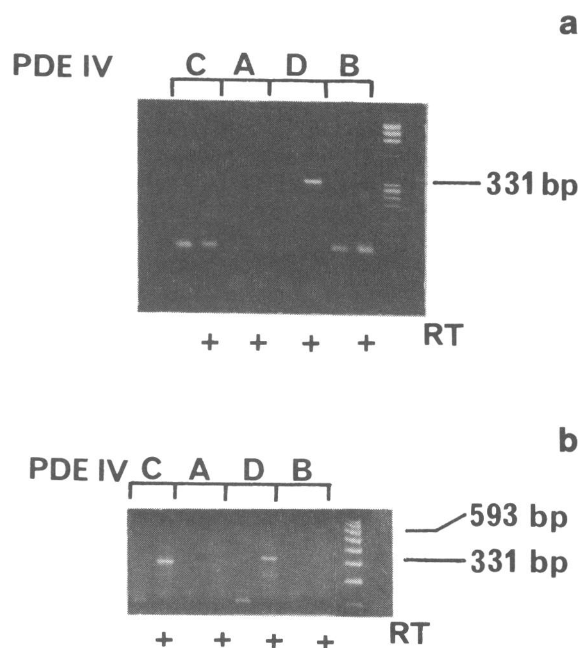

Figure 6 PDE IV message amplification phenotyping in eosinophils and smooth muscle. RNA from guinea-pig eosinophils (a) and pig aortic smooth muscle cells (Souness *et al.*, 1992a) (b) was extracted from 10^6 cells. First strand cDNA synthesis and PCR amplification (55 cycles) (using primers recognizing unique sequences in PDE IV_{A-D} (rpdes 1-4)) of total RNA from eosinophils and pig aortic smooth muscle cells were performed as described in the Methods section.

corresponding to PDE IVs_{A-D} were detected. Ethidium bromide staining of gels of RT-PCR products of guinea-pig eosinophil total RNA revealed an amplified cDNA fragment corresponding to that predicted for PDE IV_D (predicted fragment size: 331 bases) (Figure 6a). Sequencing confirmed that this PCR product corresponded to PDE IV_D. No RT-PCR-amplified products of the correct molecular sizes were detected employing primers recognizing sequences in PDE IV_A, PDE IV_B or PDE IV_C. RT-PCR of total RNA from pig aortic smooth muscle cells (using rat primers) demonstrated PDE IV_D to be the major subtype expressed (Figure 6b). A faint band corresponding to PDE IV_B was also detected. The PCR fragment detected in lane 2 of Figure 6b did not correspond to the predicted size for PDE IV_C. The only RT-PCR fragment detected employing poly (A^+) mRNA from rat smooth muscle (Clontech) corresponded to PDE IV_D (data not shown).

Inhibition of methacholine-induced contraction of guinea-pig trachealis

RP 73401 ($-\log IC_{50}$ [M] $= 7.53 \pm 0.12$, $n = 6$) was about 3 fold more potent than rolipram ($-\log IC_{50}$ [M] $= 7.01 \pm 0.04$, $n = 6$) in reversing methacholine-induced contractions of guinea-pig trachealis.

Discussion

RP 73401 is a very potent and selective inhibitor of cyclic AMP hydrolysis. It inhibits PDE IV with a very high potency and displays great selectivity over PDEs I, II, III and V. Rolipram was considerably less selective. PDE IV is the predominant isoenzyme regulating cyclic AMP levels and (consequently) function in a wide-range of inflammatory cells (Torphy & Undem, 1991) including eosinophils (Dent *et al.*, 1991; Souness *et al.*, 1991). RP 73401 suppresses eosinophil function *in vitro* and exhibits anti-inflammatory activity *in vivo* (Raeburn *et al.*, 1994), effects that can be attributed to elevation of cyclic AMP through inhibition of PDE IV.

Blood and lung eosinophil numbers are elevated in asthma and may correlate with the severity of the disease (Kay, 1985; Barnes *et al.*, 1988). The cytotoxic potential of the eosinophil is due to its ability to generate reactive oxygen species and cationic proteins which can induce epithelial damage and increase airways reactivity as demonstrated in experimental animals *in vivo* (Kay, 1985; Barnes *et al.*, 1988). We and others (Dent *et al.*, 1991; Souness *et al.*, 1991) have previously shown that PDE IV inhibitors, such as rolipram, suppress oxygen radical formation. We now show that RP 73401 was 7 fold more potent than rolipram in blocking this parameter of the eosinophil's cytotoxic armoury. More importantly, however, RP 73401 potently inhibited ECP and MBP release from eosinophils further indicating the anti-inflammatory potential of this compound. These cytotoxic proteins are associated with eosinophil-mediated damage of the respiratory mucosa, in particular epithelial disruption and shedding (Barnes *et al.*, 1988). The potent dampening effects of RP 73401 on eosinophil function may be one explanation for the potent anti-inflammatory properties of RP 73401 in guinea-pigs and rats (Raeburn *et al.*, 1994). Furthermore, the inhibition of platelet-activating factor-induced airway hyper-responsiveness in guinea-pigs by RP 73401 (Raeburn *et al.*, 1994) may also be related to suppression of MBP and ECP release.

Interesting differences were observed between the inhibitory actions of RP 73401 and rolipram against eosinophil PDE IV; in particular, solubilization or V/GSH-treatment of membrane-bound eosinophil PDE IV increases (10 fold) the potency of rolipram but leaves that of RP 73401 unaffected. The basis for this disparity is uncertain, although the demonstration by molecular biology studies (Swinnen *et al.*, 1989; Torphy *et al.*, 1992b; McLaughlin *et al.*, 1993) of four PDE IV genes may offer one possible explanation. If multiple PDE IV subtypes exist in eosinophils against which rolipram displays different affinities, the effects of solubilization and V/GSH treatment could be rationalized if these treatments selectively activated a subtype for which rolipram is more potent. If this assertion is correct, RP 73401, in contrast to rolipram, would not discriminate between the two subtypes. Our RT-PCR data do not support this hypothesis since only mRNA for PDE IV_D was detected in these cells.

An alternative hypothesis (Souness & Scott, 1993), proposes that solubilization or V/GSH treatment induces a conformational change in PDE IV which increases the potency

of rolipram. Under these conditions, there is a very strong correlation between the potency order of compounds in inhibiting catalytic activity and displacing high-affinity [^{3}H]-rolipram from brain membranes (Souness & Scott, 1993). Although high-affinity rolipram binding is known to be localized on PDE IV (Torphy *et al.*, 1992b; McLaughlin *et al.*, 1993), its nature and relevance is at present unclear. Solubilization and V/GSH-treatment induces marked potency shifts only in the compounds (rolipram, denbufylline, Ro 20-1724) with IC_{50} values in displacing [^{3}H]-($\pm$)-rolipram from PDE IV lower than those for inhibition of cyclic AMP hydrolysis (Souness *et al.*, 1992b; Souness & Scott, 1993). Conversely, no potency shifts occur with compounds (dipyridamole, trequinsin) that are more potent inhibitors of PDE IV activity than of [^{3}H]-($\pm$)-rolipram binding (Torphy *et al.*, 1992b; Souness *et al.*, 1992b). RP 73401 exhibited similar potencies on cyclic AMP hydrolysis (smooth muscle or inflammatory cell PDE IVs) and rolipram binding and its activity on eosinophil PDE IV was, predictably, not influenced by solubilization or V/GSH treatment.

The reason why rolipram was greater than 100 fold more potent against eosinophil PDE IV (solubilized or +V/GSH) than partially-purified PDE IV from smooth muscle, whereas RP 73401 does not discriminate between the enzymes from the two sources, is again unclear. Our finding that the predominant subtype in smooth muscle is, as in eosinophils, PDE IV_D indicates that subtype selectivity does not offer a satisfactory explanation for the disparity. Whether the alternative splicing of PDE IV_D (Sette *et al.*, 1994), which may influence subcellular localization (Shakur *et al.*, 1993), differentially affects the potency of inhibitors is an issue which has not been addressed. It should be noted, however, that in spite of the great potency difference between the two compounds against partially-purified PDE IV from smooth muscle, RP 73401 was only 2–3 fold more potent than rolipram in relaxing airways smooth muscle. This demonstrates that the functional effects of compounds are not mirrored in their inhibition of partially-purified smooth muscle PDE IV. A similar observation was made previously by Harris *et al.* (1989).

Tissue disruption, by releasing proteases which clip an amino acid sequence necessary for high-affinity binding, or chromatography procedures, by altering the conformational state of the enzyme (see Souness & Scott, 1993), may both influence the nature of the interaction of rolipram with partially-purified PDE IV and change its potency. If the native smooth muscle PDE IV is essentially similar to its counterpart in eosinophils, it is clear that, in contrast to rolipram, preparative procedures do not influence the potency of RP 73401, supporting the view that the two compounds exert their inhibitory effects through distinct interactions with the enzyme. Kinetic analysis of the data is not helpful in further elucidating this possibility. For both RP 73401 (Souness *et al.*, unpublished data) and rolipram (Torphy & Cieslinski, 1990), the kinetics of inhibition against partially, purified smooth muscle PDE IV are competitive whereas this is clearly not the case for either compound against the solubilized eosinophil PDE IV.

RP 73401 is 3 to 14 fold more potent than rolipram in increasing cyclic AMP accumulation and inhibiting eosinophil function ($.O_2^-$, MBP, ECP). These whole cell responses reflect better the relative potency differences of the two compounds against the solubilized or V/GSH-stimulated enzyme (10 fold) than the untreated, eosinophil particulate PDE IV (200 fold) or the smooth muscle PDE IV (3000 fold). It seems probable, therefore, that the native eosinophil (and smooth muscle) PDE IV exists in a form similar to the solubilized or V/GSH-treated enzyme.

In conclusion, RP 73401 is a potent and selective inhibitor of PDE IV and will be a useful investigative tool for elucidating the intracellular regulatory roles of PDE IV. RP 73401 increases cyclic AMP levels and potently inhibits eosinophil functions *in vitro*. Because of the importance of eosinophils in airway inflammation, our findings suggest that RP 73401 may be a promising new drug for the treatment of airway disease such as asthma. Based on the pharmacological data presented in this paper, it is likely that RP 73401 and rolipram exert their effects on cyclic AMP hydrolysis through distinct interactions with PDE IV.

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References

ASHTON, M., COOK, D.C., FENTON, G., KARLSSON, J.-A., PALFREYMAN, M.N., RAEBURN, D., RATCLIFFE, A.J., SOUNESS, J.E., THURAIRATNAM, S. & VICKER, N. (1994). Selective type IV phosphodiesterase inhibitors as anti-asthmatic agents. The synthesis and biological activities of 3-cyclopentyloxy-4-methoxybenzamides and analogues. *J. Med. Chem.*, **37**, 1696–1703.

BARNES, P.J., CHUNG, K.F. & PAGE, C.P. (1988). Inflammatory mediators and asthma. *Pharmacol. Rev.*, **40**, 49–84.

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.

DAVIES, G.E. & EVANS, D.P. (1973). Studies with two new phosphodiesterase inhibitors (ICI 58,301 and ICI 63,197) on anaphylaxis in guinea-pigs, mice and rats. *Int. Arch. Allergy Appl. Immunol.*, **45**, 467–478.

DENT, G., GIEMBYCZ, M.A., RABE, K.F. & BARNES, P.J. (1991). Inhibition of eosinophil cyclic nucleotide PDE activity and opsonised zymosan-stimulated respiratory burst by 'type-IV'-selective PDE inhibitors. *Br. J. Pharmacol.*, **103**, 1339–1346.

EPSTEIN, P.M. & HACHISU, R. (1984). Cyclic nucleotide phosphodiesterase in normal and leukemic human lymphocytes and lymphoblasts. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.*, **16**, 303–324.

FONTEH, A.N., WINKLER, J.D., TORPHY, T.J., HERAVI, J., UNDEM, B.J. & CHILTON, F.H. (1993). Influence of isoproterenol and phosphodiesterase inhibitors on platelet-activating factor biosynthesis in the human neutrophil. *J. Immunol.*, **151**, 339–350.

GIEMBYCZ, M.A. (1992). Could isoenzyme-selective phosphodiesterase inhibitors render bronchodilator therapy redundant in the treatment of bronchial asthma? *Biochem. Pharmacol.*, **43**, 2041–2051.

GIEMBYCZ, M.A. & BARNES, P.J. (1991). Selective inhibition of high-affinity Type IV cyclic AMP phosphodiesterase in bovine trachealis by AH-21-132: relevance to the spasmolytic and anti-spasmogenic actions of AH-21-132 in the intact tissue. *Biochem. Pharmacol.*, **42**, 663–677.

GLEICH, G.J., LOEGERING, D.A. & MALDONADO, J.E. (1973). Identification of a major basic protein in guinea-pig eosinophil granules. *J. Exp. Med.*, **137**, 1459–1471.

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 Km cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **251**, 199–206.

HUNT, T.C., SUMMERS, J.A., CAMPOS, M.G., RIMMER, S.J., STURTON, G., PALFAI, S. & CHURCH, M.K. (1993). Monoclonal antibodies specific for guinea-pig eosinophil major basic protein: their use in an ELISA, immunocytochemistry and flow cytometry. *Clin. Exp. Allergy*, **23**, 425–434.

KAY, A.B. (1985). Eosinophils as effector cells in immunity and hypersensitivity disorders. *Clin. Exp. Immunol.*, **62**, 1–12.

McLAUGHLIN, M.M., CIESLINSKI, L.B., BURMAN, M., TORPHY, T.J. & LIVI, G.P. (1993). A low-Km, rolipram-sensitive, cAMP-specific phosphodiesterase from human brain. Cloning and expression of cDNA, biochemical characterisation of recombinant protein, and tissue distribution of mRNA. *J. Biol. Chem.*, **268**, 6470–6476.

PEACHELL, P.T., UNDEM, B.J., SCHLEIMER, R.P., MACGLASHAN, D.W. Jr., LICHTENSTEIN, L.M., CIESLINSKI, L.B. & TORPHY, T.J. (1992). Preliminary identification and role of phosphodiesterase isozymes in human basophils. *J. Immunol.*, **148**, 2503–2510.

RAEBURN, D. & KARLSSON, J.-A. (1993). Effects of isoenzyme-selective inhibitors of cyclic nucleotide phosphodiesterase on microvascular leak in guinea pig airways *in vivo*. *J. Pharmacol. Exp. Ther.*, **267**, 1147–1152.

RAEBURN, D. & LEWIS, S. (1991). Rolipram inhibits PAF-induced bronchial hyperreactivity (BHR) in the guinea-pig in vivo: comparison with theophylline and salbutamol. *Eur. Resp. J.*, **4** (suppl. 14), 307S.

RAEBURN, D., SOUNESS, J.E., TOMKINSON, A. & KARLSSON, J.-A. (1993). Isozyme-selective cyclic nucleotide phosphodiesterase inhibitors: biochemistry, pharmacology and therapeutic potential in asthma. *Prog. Drug Res.*, **40**, 9–32.

RAEBURN, D., UNDERWOOD, S.L., LEWIS, S.A., WOODMAN, V.R., BATTRAM, C.H., TOMKINSON, A., SHARMA, S., JORDAN, R., SOUNESS, J.E., WEBBER, S.E. & KARLSSON, J.-A. (1994). Anti-inflammatory and bronchodilator properties of RP 73401, a novel and selective phosphodiesterase type IV inhibitor. *Br. J. Pharmacol.*, **113**, 1423–1431.

ROBICSEK, S.A., BLANCHARD, D.K., DJEU, J.Y., KRZANOWSKI, J.J., SZENTIVANYI, A. & POLSON, J.B. (1991). Multiple high-affinity cAMP-phosphodiesterases in human T-lymphocytes. *Biochem. Pharmacol.*, **42**, 869–877.

SCHADE, F.U. & SCHUDT, C. (1993). The specific type III and IV phosphodiesterase inhibitor zardavarine suppresses formation of tumor necrosis factor by macrophages. *Eur. J. Pharmacol.*, **230**, 9–14.

SCHNEIDER, H.H., SCHMIECHEN, R., BREZINSKI, M. & SEIDLER, J. (1986). Stereospecific binding of the antidepressant rolipram to brain protein structures. *Eur. J. Pharmacol.*, **127**, 105–115.

SEMMLER, J., WACHTEL, H. & ENDRES, S. (1993). The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor-α production by human mononuclear cells. *Int. J. Immunopharmacol.*, **15**, 409–413.

SETTE, C., VICINI, E. & CONTI, M. (1994). The rat PDE3/IVd phosphodiesterase gene codes for multiple proteins differentially activated by cAMP-dependent protein kinase. *J. Biol. Chem.*, **269**, 18271–18274.

SHAKUR, Y., PRYDE, J.G. & HOUSLAY, M.D. (1993). Engineered deletion of the unique N-terminal domain of the cyclic AMP-specific phosphodiesterase RD1 prevents plasma membrane association and the attainment of enhanced thermostability without altering its sensitivity to inhibition by rolipram. *Biochem. J.*, **292**, 677–686.

SOUNESS, J.E., CARTER, C.M., DIOCEE, B.K., HASSALL, G.A., WOOD, L.J. & TURNER, N.C. (1991). Characterization of guinea-pig eosinophil phosphodiesterase activity. Assessment of its involvement in regulating superoxide generation. *Biochem. Pharmacol.*, **42**, 937–945.

SOUNESS, J.E., HASSALL, G.A. & PARROTT, D.P. (1992a). Inhibition of pig aortic smooth muscle cell DNA synthesis by selective type III and type IV cyclic AMP phosphodiesterase inhibitors. *Biochem. Pharmacol.*, **44**, 857–866.

SOUNESS, J.E., MASLEN, C. & SCOTT, L.C. (1992b). Effects of solubilization and vanadate/glutathione complex on inhibitor potencies against eosinophil cyclic AMP-specific phosphodiesterase. *FEBS Lett.*, **302**, 181–184.

SOUNESS, J.E. & SCOTT, L.C. (1993). Stereospecificity of rolipram actions on eosinophil cyclic AMP-specific phosphodiesterase. *Biochem. J.*, **291**, 389–395.

SOUNESS, J.E., THOMPSON, W.J. & STRADA, S.J. (1985). Adipocyte cyclic nucleotide phosphodiesterase activation by vanadate. *J. Cyclic Nucleotide Protein Phosphorylation Res.*, **10**, 383–396.

SOUNESS, J.E., VILLAMIL, M.E., SCOTT, L.C., TOMKINSON, A., GIEMBYCZ, M.A. & RAEBURN, D. (1994). Possible role of cyclic AMP phosphodiesterase in the actions of ibudilast on eosinophil thromboxane generation and airways smooth muscle tone. *Br. J. Pharmacol.*, **111**, 1081–1088.

SWINNEN, J.V., JOSEPH, D.R. & CONTI, M. (1989). Molecular cloning of rat homologues of the Drosophilia melanogaster dunce cAMP phosphodiesterase: evidence for a family of genes. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5325–5329.

THOMPSON, W.J., TERASAKI, W., EPSTEIN, P.M. & STRADA, S.J. (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. *Adv. Cyclic Nucleotide Res.*, **10**, 69–92.

TORPHY, T.J. & CIESLINSKI, L.B. (1990). Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol. Pharmacol.*, **37**, 206–214.

TORPHY, T.J., LIVI, G.P., BALCAREK, J.M., WHITE, J.R., CHILTERN, F.H. & UNDEM, B.J. (1992a). Therapeutic potential of isozyme-selective phosphodiesterase inhibitors in the treatment of asthma. *Adv. Second Messenger Phosphoprotein Res.*, **25**, 289–305.

TORPHY, T.J., STADEL, J.M., BURMAN, M., CIESLINSKI, L.B., McLAUGHLIN, M.M., WHITE, J.R. & LIVI, G.P. (1992b). Co-expression of human cAMP-specific phosphodiesterase activity and high-affinity rolipram binding in yeast. *J. Biol. Chem.*, **267**, 1798–1804.

TORPHY, T.J. & UNDEM, B.J. (1991). Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax*, **46**, 512–523.

UNDERWOOD, D., OSBORN, R.R., NOVAK, L.B., MATTHEWS, J.K., NEWSHOLME, S.J., UNDEM, B.J., HAND, J.M. & TORPHY, T.J. (1993). Inhibition of antigen-induced bronchoconstriction and eosinophil infiltration in the guinea-pig by the cyclic AMP-specific phosphodiesterase inhibitor, rolipram. *J. Pharmacol. Exp. Ther.*, **266**, 306–313.

WASSOM, D.L. LOEGERING, D.A. & GLEICH, G.J. (1979). Measurement of guinea-pig eosinophil major basic protein by radioimmunoassay. *Mol. Immunol.*, **16**, 711–719.

WRIGHT, C.D., KUIPERS, P.J., KOBYLARZ-SINGER, D., DEVALL, L.J., KLINKEFUS, B.A. & WEISHAR, R.E. (1990). Differential inhibition of human neutrophil functions. Role of cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase. *Biochem. Pharmacol.*, **40**, 699–707.

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