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Evidence that cyclic AMP phosphodiesterase inhibitors suppress TNFα generation from human monocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer

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1 We have investigated the inhibitory effects of RP 73401 (piclamilast) and rolipram against human monocyte cyclic AMP-specific phosphodiesterase (PDE4) in relation to their effects on prostaglandin (PG)E_2-induced cyclic AMP accumulation and lipopolysaccharide (LPS)-induced TNFα production and TNFα mRNA expression.

2 PDE4 was found to be the predominant PDE isoenzyme in the cytosolic fraction of human monocytes. Cyclic GMP-inhibited PDE (PDE3) was also detected in the cytosolic and particulate fractions. Reverse transcription polymerase chain reaction (RT-PCR) of human monocyte poly (A^+) mRNA revealed amplified products corresponding to PDE4 subtypes A and B of which the former was most highly expressed. A faint band corresponding in size to PDE4D was also observed.

3 RP 73401 was a potent inhibitor of cytosolic PDE4 (IC_{50}: 1.5±0.6 nM, $n=3$). (±)-Rolipram (IC_{50}: 313±6.7 nM, $n=3$) was at least 200 fold less potent than RP 73401. **R**-(−)-rolipram was approximately 3 fold more potent than **S**-(+)-rolipram against cytosolic PDE4.

4 RP 73401 (IC_{50}: 9.2±2.1 nM, $n=6$) was over 50 fold more potent than (±)-rolipram (IC_{50}: 503±134 nM, $n=6$)) in potentiating PGE_2-induced cyclic AMP accumulation. **R**-(−)-rolipram (IC_{50}: 289±121 nM, $n=5$) was 4.7 fold more potent than its **S**-(+)-enantiomer (IC_{50}: 1356±314 nM, $n=5$). A strong and highly-significant, linear correlation ($r=0.95$, $P<0.01$, $n=13$) was observed between the inhibitory potencies of a range of structurally distinct PDE4 inhibitors against monocyte PDE4 and their ED_{50} values in enhancing monocyte cyclic AMP accumulation. A poorer, though still significant, linear correlation ($r=0.67$, $P<0.01$, $n=13$) was observed between the potencies of the same compounds in potentiating PGE_2-induced monocyte cyclic AMP accumulation and their abilities to displace [^{3}H]-rolipram binding to brain membranes.

5 RP 73401 (IC_{50}: 6.9±3.3 nM, $n=5$) was 71 fold more potent than (±)-rolipram (IC_{50}: 490±260 nM, $n=4$) in inhibiting LPS-induced TNFα release from monocytes. **R**-(−)-rolipram (IC_{50}: 397±178 nM, $n=3$) was 5.2-fold more potent than its **S**-(+)- enantiomer (IC_{50}: 2067±659 nM, $n=3$). As with cyclic AMP, accumulation a closer, linear correlation existed between the potency of structurally distinct compounds in suppressing TNFα with PDE4 inhibition ($r=0.93$, $P<0.01$, $n=13$) than with displacement of [^{3}H]-rolipram binding ($r=0.65$, $P<0.01$, $n=13$).

6 RP 73401 (IC_{50}: 2 nM) was 180 fold more potent than rolipram (IC_{50}: 360 nM) in suppressing LPS (10 ng ml^{-1})-induced TNFα mRNA.

7 The results demonstrate that RP 73401 is a very potent inhibitor of TNFα release from human monocytes suggesting that it may have therapeutic potential in the many pathological conditions associated with over-production of this pro-inflammatory cytokine. Furthermore, PDE inhibitor actions on functional responses are better correlated with inhibition of PDE4 catalytic activity than displacement of [^{3}H]-rolipram from its high-affinity binding site, suggesting that the native PDE4 in human monocytes exists predominantly in a 'low-affinity' state.

Keywords: Cyclic AMP-phosphodiesterase; RP 73401; rolipram; tumour necrosis factor-α; monocytes

Introduction

The therapeutic potential of adenosine 3':5'-cyclic monophosphate (cyclic AMP)-specific phosphodiesterase (PDE4) inhibitors has attracted considerable attention, primarily because of their dampening effects on the functions of several inflammatory/immunocompetent cells (Palfreyman & Souness, 1996). Most interest to date has focused on the potential anti-asthma effects of these compounds but, more recently, the potent suppression of tumour necrosis factor-alpha (TNFα) production from mononuclear phagocytes (Schade & Schudt, 1993; Semmler *et al.*, 1993; Prabhakar *et al.*, 1994) by PDE4 inhibitors has opened the possibility of treating the wide-number of pathological conditions associated with over-elaboration of this pro-inflammatory cytokine. Indeed, over expression/production of TNFα has been shown in auto-immune diseases (arthritis, multiple sclerosis etc) (Tracey & Cerami, 1993; Feuerstein *et al.*, 1995), certain viral diseases (e.g. AIDS) (Fauci *et al.*, 1991) and bacterial or parasitic infections (e.g. septic shock, cerebral ischaemia) (Feuerstein *et al.*, 1995; Hinshaw *et al.*, 1992). Evidence has emerged demonstrating that anti-TNFα antibodies can ameliorate the symptoms in some of these human pathological conditions (Williams *et al.*, 1992). PDE4 inhibitors ameliorate disease progression in animal models of arthritis (Sekut *et al.*, 1995) and multiple sclerosis (experimental allergic encephalomyelitis) (Genain *et al.*, 1995; Sommer *et al.*, 1995) and, in the clinic, the non-selective PDE inhibitor, pentoxifylline, which lowers LPS-induced TNFα production (Waage *et al.*, 1990), reduces HIV replication (Fazely *et al.*, 1991).

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A large number of compounds of diverse structures have been synthesized which are believed to exert their anti-inflammatory effects through inhibition of PDE4 (Palfreyman & Souness, 1996); however, in some instances, poor correlations have been reported between PDE4 inhibition and functional responses (Harris *et al.*, 1989; Souness *et al.*, 1991). Indeed, certain central (Schmiechen *et al.*, 1990) and peripheral actions (Harris *et al.*, 1989; Souness & Scott, 1993) of PDE4 inhibitors are more tightly correlated with their potencies in displacing [^{3}H]-rolipram from brain membranes than inhibition of PDE4. The nature of this binding site is uncertain although it is known to be associated with at least two PDE4 subtypes (Torphy *et al.*, 1992; McLaughlin *et al.*, 1993). The rank potency order of compounds in inhibiting human recombinant (hr) PDE4A catalytic activity and displacing [^{3}H]-rolipram are markedly distinct (Torphy *et al.*, 1992). For example, the K_i of rolipram on catalysis is almost 100 fold lower than its K_{iapp} in the binding assay (Torphy *et al.*, 1992). Furthermore, whereas the **R**-(−)-enantiomer of rolipram is only 3 fold more potent than the **S**-(+)-enantiomer in inhibiting cyclic AMP hydrolysis, 20 fold stereoselectivity is observed for binding (Torphy *et al.*, 1992). One hypothesis proposes that the high-affinity rolipram binding site (HARBS) represents a site distinct from the catalytic site whose influence on catalysis is determined by the conformational state of PDE4 (Souness *et al.*, 1992; Souness & Scott, 1993); however, the requirement for two sites has been questioned (Torphy, 1994) and HARBS may simply reflect a high-affinity interaction of rolipram with one (or more) of multiple conformational states of PDE4.

Recent studies demonstrate that potentiation of isoprenaline induced cyclic AMP accumulation in guinea-pig intact eosinophils by PDE inhibitors with diverse structures is better correlated with their affinities for HARBS than their potencies against the membrane-bound, eosinophil PDE4 (Souness & Scott, 1993). For example, trequinsin, which is approximately equipotent with rolipram against PDE4, is 100 fold less potent in increasing cyclic AMP accumulation (Souness *et al.*, 1991) and its affinity for HARBS is over 100 fold less than that of rolipram (Souness & Scott, 1993). Marked stereoselectivity, which is a feature of rolipram's interaction with HARBS but not with untreated PDE4, is observed in its potentiation of eosinophil cyclic AMP accumulation (Souness & Scott, 1993). Finally, RP 73401 (piclamilast), which is 200 fold more potent than rolipram against untreated esoinophil PDE4, is only 3–4 fold more potent in elevating cyclic AMP levels in intact eosinophils, a potency difference similar to that observed on HARBS (Souness *et al.*, 1995).

We have investigated the inhibitory effects of RP 73401 (Ashton *et al.*, 1994; Souness *et al.*, 1995), rolipram and several other structurally diverse PDE inhibitors on LPS-induced TNFα release and PGE_2-induced cyclic AMP accumulation to determine whether their effects on intact, human monocytes functions are more closely associated with inhibition of PDE4 or displacement of [^{3}H]-rolipram from its binding site in brain membranes. The results demonstrate that, in contrast to guinea-pig eosinophils, PDE inhibitor actions on human monocyte functional responses are better correlated with PDE4 catalytic activity than with displacement of [^{3}H]-rolipram from HARBS suggesting that PDE4 exists predominantly in a 'low-affinity' state in this cell-type.

Methods

Preparation of human peripheral blood monocytes

Freshly drawn blood from normal healthy donors was mixed (4:1, v/v) with sodium citrate (3.8% w/v). Mononuclear cells were prepared by centrifugation of the blood on Histopaque-1077 (Sigma Diagnostics) according to the manufacturers' instructions. The fraction enriched with mononuclear cells was washed and resuspended in Hank's balanced salts solution (HBSS) supplemented with deoxyribonuclease (37.5 u/ml^{-1}) and human serum albumin (0.3%). Differential (cytospin) cell counts revealed that the mononuclear cell fraction routinely comprised 70–80% monocytes.

Cells from the mononuclear leukocyte fraction were centrifuged (200 *g*, 10 min, 20°C), resuspended, at a density of 10^6 cells ml^{-1}, in RPMI 1640 containing foetal calf serum (FCS) (1%), penicillin (50 u ml^{-1}) and streptomycin (50 μg ml^{-1}) and allowed to adhere (96 well plates for TNFα assays, 24 well plates for cyclic AMP and cytokine mRNA assays, 250 ml culture flasks for PDE studies). Following incubation (5% CO_2, 37°C) for 90 min, medium containing non-adherent cells was removed and the cells were washed once with fresh medium. For TNFα-, cytokine mRNA- and cyclic AMP asays, fresh medium was added to the cells. For PDE studies, cells were washed twice with HBSS (0°C) before being further processed (see below).

In some PDE studies, monocytes were purified from erythrocyte-depleted blood using magnetizable polystyrene beads coated with a primary antibody specific for the CD14 membrane antigen (Dynabeads M-450 CD14) according to the manufacturer's instructions (Dynal U.K., Ltd.).

Preparation of monocyte sub-cellular fractions

Ice-cold 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-lycine-chloromethylketone (TLCK), 20 μM, leupeptin 10 μg ml^{-1}, aprotinin, 2000 u ml^{-1}) was added to adherent cells ($30-40 \times 10^6$) which had been washed twice with HBSS. The cells were scraped from the surface of the culture flask and homogenized with a Dounce homogenizer (20 strokes). The homogenate was centrifuged (105 000 *g*, 60 min), the supernatant was collected and the pellet resuspended in an equal volume of homogeniziation buffer. In experiments in which monocytes were purified using CD14 coated beads, the washing and homogenization steps were conducted on the bead-attached cells.

Measurement of PDE activity

PDE activity was determined by the two-step radioisotope method of Thompson *et al.* (1979). The reaction mixture contained: Tris-HCl 20 mM (pH 8.0), $MgCl_2$ 10 mM, 2-mercaptoethanol 4 mM, ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) 0.2 mM, 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.

Categorization of PDE isoenzymes

The nomenclature adopted in this paper for the different cyclic nucleotide PDEs is based on that of Beavo & Reifsnyder (1990). PDE4 substrates referred to in this paper are based on human cDNA nucleotide sequences (PDE4A-D) as reported by Bolger *et al.* (1993), Bolger (1994) and McLaughlin *et al.* (1993).

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 were 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 monocyte cyclic AMP accumulation

For measurement of cyclic AMP, freshly prepared, adherent monocytes in 24 well plates (1×10^6 cells/well) were pre-incubated in RPMI 1640 containing foetal calf serum (FCS) (1%). To test the effects of PDE4 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 PGE_2 (1 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 monocyte TNFα release

Adherent cells in culture medium were incubated for 1 h (5% CO_2, 37°C) with fresh medium containing PDE inhibitors or vehicle (0.1% dimethylsulphoxide). LPS (10 ng ml^{-1}) was then added to the cells and the incubation continued for a further 18 h. Cell supernatants were removed into 96 well, 0.22 μm filtration plates for storage at −70°C.

TNFα concentrations in cell supernatants were quantified by sandwich ELISA. Briefly, ELISA plates were coated with 3 μg ml^{-1} of polyclonal goat anti-human TNFα antibody in bicarbonate buffer (pH 9.9). Monocyte supernatant samples or human recombinant TNFα standards were vacuum filtered into the corresponding wells of the ELISA plate. Rabbit polyclonal anti-human TNFα antibody (3 μg ml^{-1}) was used as the second antibody and polyclonal goat anti-rabbit IgG coupled to horseradish peroxidase was used as the detection antibody. The peroxidase substrate was 3,3′,5′,5′-tetramethylbenzidine (TMB), in the presence of hydrogen peroxide.

TNFα concentrations in supernatants from control and LPS-stimulated monocyte incubations were calculated by interpolation from a standard (log/log) curve (0.125–16 ng ml^{-1}) fitted by linear regression using a Multicalc software program (Wallac U.K., Ltd.).

Measurement of monocyte cytokine mRNA expression

Monocytes were prepared and incubated as described above for the measurement of TNFα protein. mRNA was obtained from cells (10^6) using Quickprep purification kits (Pharmacia Biotech). First strand cDNA synthesis and cDNA amplification were performed using a GeneAMP RNA PCR kit (Perkin Elmer Cetus). Primers specific for human TNFα were purchased from Perkin Elmer Cetus. The sequences of the TNFα primers are as follows: 5′-CAGAGGGAAGAGTTCCCCAG-3′ (sense), 5′-CCTTGGTCTGGTAGGAGACG-3′* (antisense) (product size: 325). PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin Elmer Cetus) set for 35 cycles. Primer fragments were analysed by electrophoresis on 2% agarose gels, and DNA was visualized by ethidium bromide staining.

The Quant-Amp assay system (Amersham, England) was employed for semi-quantification of PCR products. Briefly, PCR was conducted with the primers detailed above, one of which was biotinylated (denoted with asterisk above), in the presence of [^{3}H]-dTTP. Upon completion of the ampification cycles, streptavidin-coated SPA (scintillation proximity assay) fluomicrospheres (SPA beads) were added to capture the biotinylated PCR products. The tritiated nucleotides incorporated into amplified product (proportional to the amount of amplified product) was measured by scintillation spectroscopy (LKB/Wallac 1219 Rackbeta).

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

(±)-Rolipram was brominated in-house in CCl_4 (Mr Kenneth Clow, Discovery Chemistry Department, Rhône-Poulenc Rorer) and subsequently tritiated by catalytic reduction with palladium and charcoal by Amersham International. 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 PDE4 subtypes

mRNA was obtained from adherent monocytes (10^6) using Quickprep purification kits (Pharmacia Biotech). First strand cDNA synthesis and cDNA amplification were performed using a GeneAMP RNA PCR kit (Perkin Elmer Cetus). Primers specific for the different human PDE4 subtypes were designed by Mr I. Giddings (Molecular Medicine Unit, King's College, London) based on reported sequences (Bolger *et al.*, 1993; McLaughlin *et al.*, 1993) from the Genbank data-base. The primer sequences are as follows: PDE4A, 5′-GTGGGGGCTGGGGAATGA-3′,5′-GAAGGGAAAGGG-GATGGAGAGT-3′ (product size: 601), which correspond to the sense sequence from base 2972 to base 2989 and the antisense sequence from base 3551 to base 3572 of HSPDE4A5 (clone PDE46), respectively; PDE4A, 5′-TGGTG-CTTCTGCCTTTAGTTTTAG -3′, 5′ - CCCGTGCTCCTTC-ATTATTTG-3′ (product size: 238) which correspond to the sense sequence from base 59 to base 82 and the antisense sequence from base 276 to base 296 of HSPDE4B2A (clone hb-PDE1); PDE4C 5′-GAGGGGGAAGAGACAGCTTTAGC-3′, 5′-CACTGGAGAGGGTGGACTAGAGG-3′ (product size: 437) which corresponds to the sense sequence from base 647 to base 669 and the antisense sequence from base 1061 to base 1083 of HSPDE4C1 (clone PDE21); PDE4D, 5′-ATTGCCCACGATAGCTGCTCAA-3′, 5′-GGTCATAAT-CGCTGTCGGATCG-3′ (product size: 300) which corresponds to the sense sequence from base 25 to base 46 and the antisense sequence from base 303 to base 324 of HSPDE4D3 (clone PDE43). Primers for the house-keeping gene, hypoxanthine phosphoribosyl transferase (HPRT) (Stratagene) were used routinely to confirm the integrity of the mRNA preparation. The sequences of the HRPT primers are: 5′-GTAATGATCAGTCAACGGGGGAC-3′ (sense), 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ (antisense) (product size: 176). PCR amplification was performed using 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 analyzed by electrophoresis on 2% agarose gels, and DNA was visualized by ethidium bromide staining. Each set of primers was first tested using human brain poly (A^+) mRNA (Clontech). Agarose gel electrophoresis of ethidium bromide-stained DNA fragments amplification showed that there was one DNA fragment in each reaction corresponding to the size of the expected PDE4 subtype fragment as defined by the primers used in the PCR.

Materials

RP 73401 [N-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide], denbufylline (1,3-di-n-butyl-7-[2′oxopropyl]-xanthine, BRL 30892), rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone], compound A (N-(1-oxido-3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide), compound B (N-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-(methylthio)benzamide), compound C ((±)N-(3,5-dichloropyrid-4-yl)-3-cyclopent-2-enyloxy-4-methoxybenzamide) and compound D (N-(2,6-dichlorophenyl)-3-cy-

clopentyloxy-4-methoxybenzamide) were synthesized by the department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd (Dagenham, Essex, U.K.). Ibudilast (3-isobutyryl-2-isopropylpryrazolo [1,5-a]pyridine, KC-404) was a gift from Kyorin Pharmaceutical Co. Ltd., (Chiyoda-ku, Tokyo, Japan). Ro 20-1724 [1-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was obtained from Roche Products Ltd (Welwyn Garden City, U.K.). Siguazodan (2-cyano-1-methyl-3-[4-methyl-6-oxo-1,4,5,6-tetrahydro-pyridazin-3-yl-phenyl]guanidine, SK&F 94836) was a generous gift from Smith Kline Beecham Ltd (Welwyn Garden City, U.K.). Trequinsin (9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2H-pyrimido[6,1-a]isoquinolin-4-one, HL-725) and polyclonal goat anti-rabbit IgG horseradish peroxidase was purchased from Calbiochem-Novabiochem U.K. Ltd (Nottingham, U.K.). The enantiomers of (±)-rolipram were separated as described by Schneider *et al.* (1986). [Methyl, 1′,2′-^{3}H]thymidine 5′-triphosphate ([^{3}H]-dTTP] (90–130 Ci mmol^{-1}), 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. Recombinant human TNFα and polyclonal goat anti-human TNFα antibody were obtained from R&D Systems Ltd (Abingdon, U.K.). Polyclonal rabbit anti-human TNFα antibody was from Endogen Inc. (Boston, U.S.A.). Cell culture reagents were from Gibco BRL (Paisley, Scotland), tissue culture plates (Nunc) from Life Technologies Ltd (Paisley, U.K.), immunoplates from Costar (High Wycombe, U.K.) and 96-well infiltration (0.22 μm) plates from Millipore, U.K. Ltd (Harrow, U.K.). The Quickprep mRNA purification kits were purchased from Pharmacia Biotech (St Albans, 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.).

Statistical analysis

To investigate whether statistically significant ($P<0.05$) relationships existed between inhibition of TNFα release or stimulation of cyclic AMP accumulation by several standard and Rhône-Poulenc Rorer compounds with their inhibition of PDE4 or displacement of [^{3}H]-rolipram from brain membranes, determination of linear (r) or rank order correlations (Spearman's rho) of the respective $\log_{10}$M values were conducted using the RS/1 programme (BBN Software Products Corporation, Cambridge, MA, U.S.A.).

Results

Monocyte cyclic AMP PDE

Following gentle homogenization of monocytes and centrifugation of the homogenate at 105 000 g, 60% the cyclic AMP PDE activity was associated with the cytosolic fraction. Endogenous regulators (cyclic GMP, Ca^{2+}/calmodulin) as well as isoenzyme-selective inhibitors of cyclic GMP-inhibited PDE (PDE3) (siguazodan) (Murray *et al.*, 1990) and PDE4 (rolipram) were employed to make a preliminary characterization of the isoenzyme types present in the monocyte sub-cellular fractions. Cyclic AMP hydrolytic activity in the cytosolic fraction was comprised predominantly (75%) of PDE4 although a proportion of the cytosolic (24%) and particulate (50%) activities was susceptible to inhibition by cyclic GMP (10 μM) and siguazodan (10 μM) indicating the presence of PDE3. Little rolipram inhibitable cyclic AMP PDE activity was detected in the particulate fraction. The initial observation of the presence of PDE3 activity was made in cells purified by adherence to plastic flasks. The demonstration of PDE3 in monocytes, although in agreement with a recent study (Verghese *et al.*, 1995), differs from previous reports (Thompson *et al.*, 1976; White *et al.*, 1990). We therefore investigated whether the PDE3 might originate from contaminating lymphocytes by purifying the monocytes with CD-14 coated dynabeads. Even in cells purified by this technique, a similar proportion of the cyclic AMP PDE activity was inhibited by cyclic GMP. The presence of PDE1 and PDE2 was excluded, since no stimulation of activity in response to Ca^{2+} (2 mM) plus calmodulin (10 u ml^{-1}) or cyclic GMP (10 μM) was detected. Vanadate/glutathione complex, which activates eosinophil PDE4 but stimulates cytosolic cyclic AMP PDE (42%) had no effect on the particulate enzyme.

Inhibitor effects on cyclic AMP PDE were investigated in the freshly-prepared crude cytosolic fraction to avoid the possible consequences of fractionation (Souness & Scott, 1993). Because of the PDE3 activity, PDE4 was measured in the presence of siguazodan (10 μM).

RP 73401 was a potent inhibitor of the cytosolic PDE4 (IC_{50}: 1.5±0.6 nM, $n=3$). Rolipram (IC_{50}: 313±6.7 nM, $n=3$) was at least 200 fold less potent than RP 73401 (Figure 1a). Denbufylline, Ro 20-1724, ibudilast, 3-isobutyl-1-methylxanthine (IBMX), trequinsin and dipyridamole were also considerably less potent than RP 73401 against the cytosolic PDE4; Rhône-Poulenc Rorer compounds (A-D) were slightly less potent than RP 73401 against monocyte PDE4 (Table 1). Little rolipram stereoselectivity was observed with the **R**-(−)-enantiomer (IC_{50}: 285±115 nM, $n=3$) being only approximately 3 fold more potent than the **S**-(+)-enantiomer (IC_{50}: 750±50 nM, $n=3$) (Figure 1b).

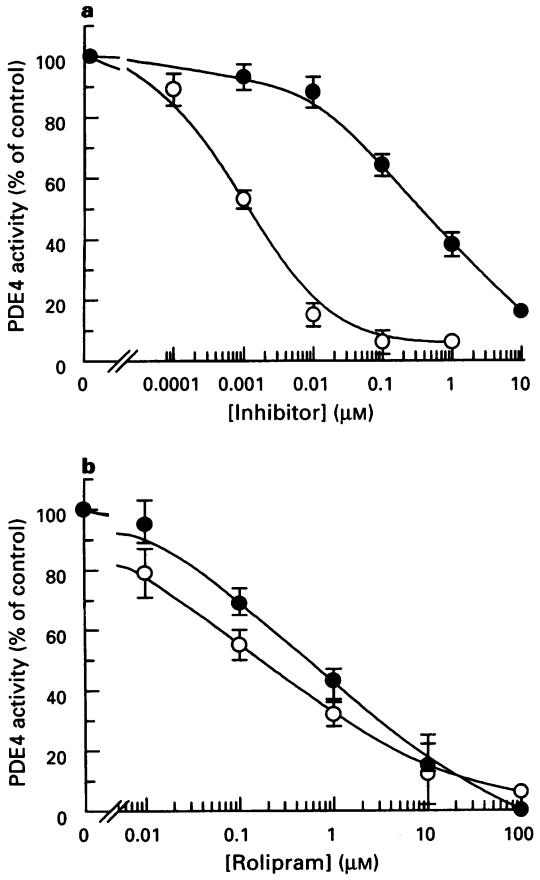

Figure 1 Inhibition of monocyte PDE4 by (a) RP 73401 (○) and (±)-rolipram (●) and (b) **R**-(−)-rolipram (○) and **S**-(+)-rolipram (●). The results represent the mean±s.e.mean of 3 experiments.

Table 1 Potencies of PDE4 inhibitors in inhibiting PDE4, displacing [^{3}H]-rolipram binding, suppressing monocyte TNFα release and increasing cyclic AMP accumulation

Compound	*PDE4 inhibition* (IC_{50}, μM)	*Displacement of [^{3}H] rolipram* (K_{iapp}, μM)	*Suppression of TNFα release* (IC_{50}, μM)	*Potentiation of cyclic AMP accumulation* (EC_{50}, μM)
RP 73401	0.0012	0.0004	0.007	0.009
R-(−)-rolipram	0.29	0.0009	0.40	0.29
S-(+)-rolipram	0.75	0.013	2.1	1.4
(±)-Rolipram	0.31	0.0017	0.49	0.50
Denbufylline	0.20	0.0041	0.20	2.0
Ro 20-1724	2.4	0.017	1.0	8.5
Ibudilast	1.3	0.010	0.30	4.4
IBMX	14	0.84	50	270
Trequinsin	0.40	1.7	10	3.0
Compound A	0.0018	0.0028	0.035	0.024
Compound B	0.0036	0.0034	0.23	0.042
Compound C	0.0022	0.0086	0.011	0.008
Compound D	0.004	0.0097	0.008	0.044

Cyclic AMP PDE activity was measured in the cytosolic fraction of human monocytes in the presence of siguazodan (10 μM). [^{3}H]-rolipram binding was measured in brain membranes. TNFα release was stimulated with LPS (10 ng ml^{-1}) and cyclic AMP synthesis stimulated with PGE_2 (10 μM). The results represent the means of 2–4 experiments.

Cyclic AMP accumulation in intact monocytes

RP 73401 (EC_{50}: 9.2±2.1 nM, $n=6$) was over 50 fold more potent than (±)-rolipram (EC_{50}: 503±134 nM, $n=6$) in potentiating prostaglandin (PG) E_2-induced cyclic AMP accumulation (Figure 2a). **R**-(−)-rolipram (EC_{50}: 289±121 nM, $n=5$) was 4.7 fold more potent than its **S**-(+)-enantiomer (EC_{50}: 1356±314 nM, $n=5$) (Figure 2b). Interestingly, trequinsin (EC_{50}: 3050±650 nM, $n=3$) was only 6 fold less potent than (±)-rolipram. Despite the presence of PDE3 in the human monocytes, siguazodan (10 μM), had no significant effect on cyclic AMP accumulation and did not enhance the actions of rolipram or PGE_2 (data not shown).

Strong linear ($r=0.95$, $P<0.01$, $n=13$) and rank (Spearman's rho = 0.93, $P<0.01$) correlations were observed between the inhibitory potencies of a range of structurally distinct PDE4 inhibitors against monocyte PDE4 and their EC_{50} values in enhancing monocyte cyclic AMP accumulation (Figure 3c; Table 1). A poorer, though still significant, linear correlation ($r=0.67$, $P<0.01$, $n=13$) was observed between the potencies of the same compounds in potentiating PGE_2-induced monocyte cyclic AMP accumulation and their abilities to displace [^{3}H]-rolipram from brain membranes (Figure 3a; Table 1). The rank correlation (Spearman's rho = 0.70, $P<0.01$) was also poorer than that observed for the association between cyclic AMP stimulation and PDE4 inhibition. We have not measured rolipram binding to human monocytes because of insufficient cells from human volunteers; however, there was very good agreement between the potencies of PDE inhibitors in displacing [^{3}H]-rolipram from guinea-pig brain membranes and human monocyte PDE4A (Torphy *et al.*, 1992) (data not shown), justifying the use of the former preparation in these studies.

TNFα release from monocytes

RP 73401 (IC_{50}: 6.9±3.3 nM, $n=5$) was 71 fold more potent than (±)-rolipram (IC_{50}: 490±260 nM, $n=4$) in inhibiting LPS-induced TNFα release from monocytes (Figure 4a). Some rolipram stereoselectivity was observed with the **R**-(−)-enantiomer (IC_{50}: 397±178 nM, $n=3$) being approximately 5 fold more potent than its **S**-(+)-enantiomer (IC_{50}: 2067±659 nM, $n=3$) (Figure 4b). In these experiments, inhibition of TNFα by PDE4 inhibitors was observed in the absence of PGE_2. In contrast, for measurable cyclic AMP accumulation to occur over a 10 min incubation period, a combination of a PDE4 inhibitor and PGE_2 are required. In spite of the different requirements for an added stimulus of adenylate cyclase, PDE inhibitor effects on the two intact-cell

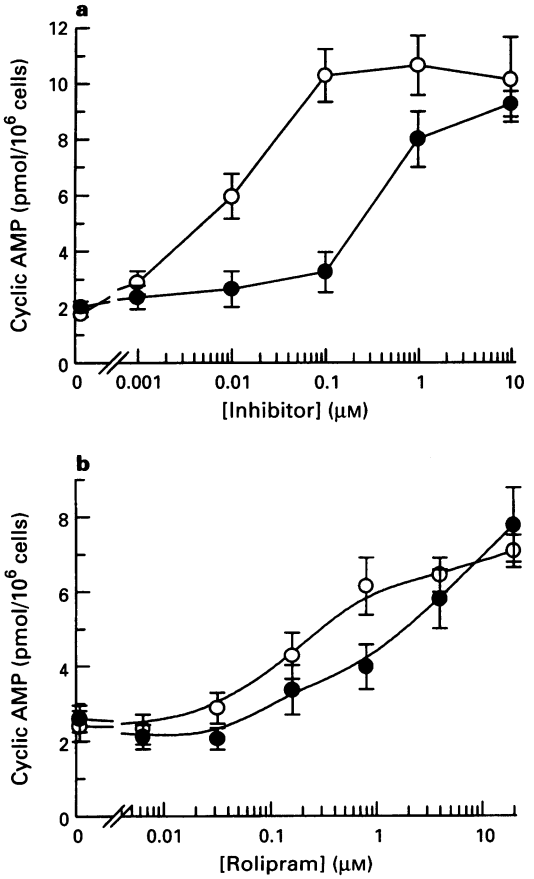

Figure 2 Potentiation of isoprenaline-induced cyclic AMP accumulation in intact monocytes by (a) RP 73401 (○) and (±)-rolipram (●) and (b) **R**-(−)-rolipram (○) and **S**-(+)-rolipram (●). The results represent the mean±s.e.mean of 3–6 experiments.

parameters were closely, linearly correlated (Figure 5, Table 1) ($r=0.94$, $P<0.01$, $n=13$; Spearman's rho = 0.85, $P<0.01$). As with cyclic AMP accumulation, a close linear correlation existed between the potency of structurally distinct compounds in suppressing TNFα with PDE4 inhibition ($r=0.93$, $P<0.01$, $n=13$) than with displacement of [^{3}H]-rolipram from brain membranes ($r=0.65$, $P<0.01$, $n=13$) (Figure 3b, d; Table 1).

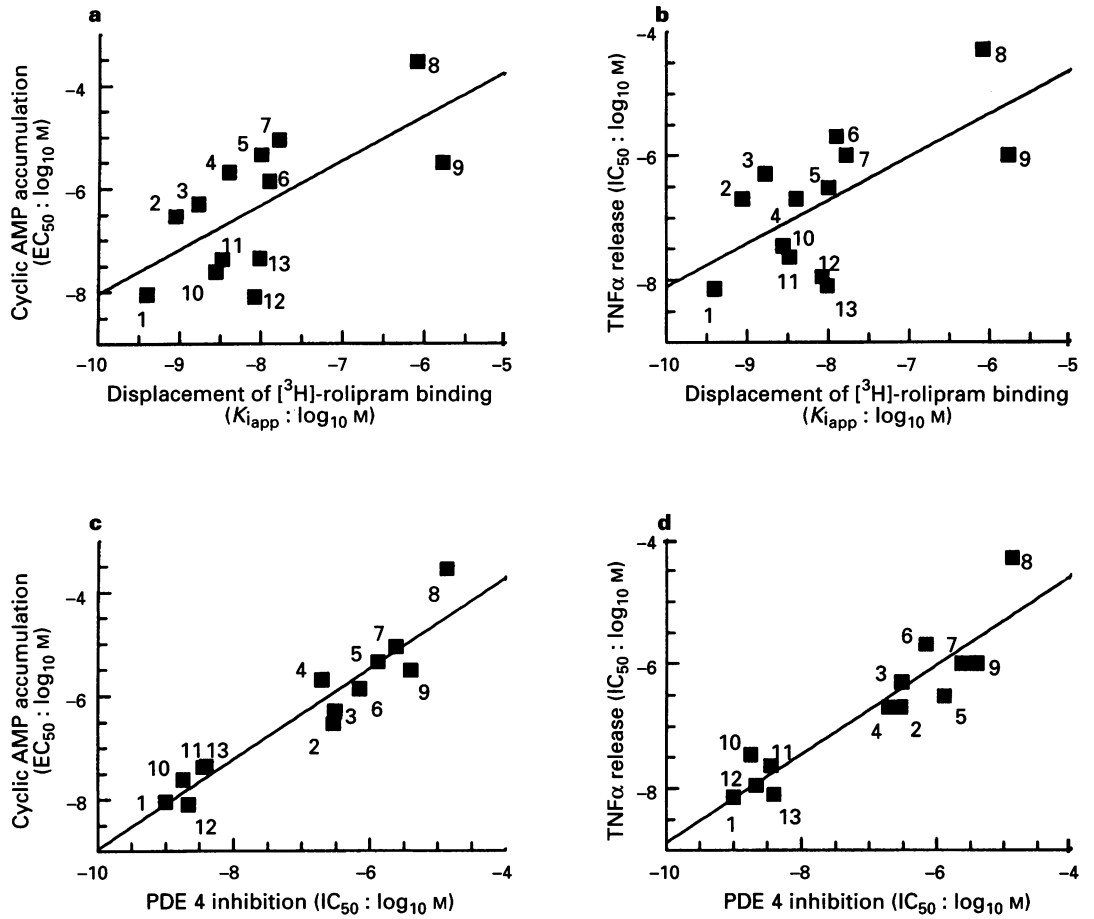

Figure 3 Displacement of [^{3}H]-rolipram binding to brain membranes (a, b) and inhibition of monocyte PDE4 (c, d) as functions of stimulation of cyclic AMP accumulation (a, c) and suppression of TNFα release (b, d). Rolipram binding data is expressed as K_{iapp} value (log concn), PDE4 data as IC_{50} values (log concn), cyclic AMP data as EC_{50} values (log concn) and TNFα data as IC_{50} values (log concn). Cyclic AMP stimulation and TNFα inhibition as a function of rolipram binding are significant (cyclic AMP, $r=0.67$, $P<0.01$, $n=13$; TNFα, $r=0.65$, $P<0.01$, $n=13$). Cyclic AMP stimulation and TNFα inhibition as a function of PDE4 inhibition are highly significant (cyclic AMP, $r=0.95$, $P<0.01$, $n=13$; TNFα, $r=0.93$, $P<0.01$, $n=13$). Compounds (1) RP 73401; (2) **R**-(−)-rolipram; (3) (±)-rolipram; (4) denbufylline; (5) ibudilast; (6) S-(+)-rolipram; (7) Ro 20-1724; (8) IBMX; (9) trequinsin; (10) compound A; (11) compound B (12) compound C; (13) compound D.

The rank correlation was tighter for TNFα inhibition and PDE4 inhibition (Spearman's rho = 0.90, $P<0.01$) than with displacement of [^{3}H]-rolipram binding (Spearman's rho = 0.66, $P<0.05$). As with cyclic AMP accumulation, siguazodan (10 μM) did not affect TNFα release in either the absence or presence of RP 73401 (data not shown).

Cytokine mRNA expression

RP 73401 (IC_{50}: 2 nM) was 180 fold more potent than rolipram (IC_{50}: 360 nM) in suppressing LPS (10 ng ml^{-1})-induced TNFα mRNA expression (Figure 6). RP 73401 did not affect the LPS-induced expression of IL-1α, IL-1β and IL-6 (data not shown).

Expression of PDE4 subtype mRNA expression

RT-PCR was used to determine the PDE4 subtypes expressed in monocytes. Primers designed to recognize sequences in the genes of 4 different human PDE4 subtypes (PDE4A-D) (Bolger *et al.*, 1993; McLaughlin *et al.*, 1993) were employed in these studies. Each set of primers was first tested employing reverse transcribed human brain -poly (A^+) mRNA (Clontech) from which PCR amplified cDNA fragments of the correct size corresponding to PDE4 subtypes A, B and D were detected. Ethidium bromide staining of gels of RT-PCR products of monocyte poly (A^+) mRNA revealed amplified products corresponding to PDE4 subtypes A and B of which the former was most highly expressed (Figure 7). A faint band corresponding in size to PDE4D was also observed. Previous studies (Verghese *et al.*, 1995), indicated very low expression of PDE4 subtype mRNA expression in monocytic cells (Mono mac 6 cells), although expression was increased by exposure to dibutryl cyclic AMP. In our RT-PCR studies, high cycle numbers were required to detect bands corresponding to PDE4 subtypes, indicating very low expression of mRNA in these monocytes.

Discussion

RP 73401 is a very potent inhibitor of TNFα release from human monocytes. Although this compound can inhibit TNFα release in the absence of a measurable accumulation of cyclic AMP, the close correlation between the potency order of PDE inhibitors of diverse structures on the two parameters suggests a causal relationship. In other cell-types, activation of cyclic AMP-dependent protein kinase A (PKA) by PDE4 inhibitors occurs in the absence of a measurable accumulation of cyclic AMP (Souness *et al.*, 1991). The inhibitory effect of RP 73401 on TNFα protein release appears to be a consequence of suppression of mRNA expression. As with other PDE4 inhibitors (Prabhakar *et al.*, 1994), the effect of RP 73401 on LPS-induced cytokine mRNA expression is specific for TNFα with interleukin-1α (IL-1α), IL-1β and IL-6 mRNA being unaffected. The exact site of action of cyclic AMP in regulating

TNFα mRNA expression is uncertain, although the recent demonstration of an AP-1/CRE-like promoter sequence in the human TNFα gene suggests that PKA-dependent regulation of cyclic AMP-responsive transcription factors may be involved (Newell *et al.*, 1994). Evidence has recently emerged suggesting that cyclic AMP can attenuate activation of NF-κB (Satriano & Schlondorf, 1994), another transcription factor implicated in LPS-induced stimulation of TNFα expression (Ishikawa *et al.*, 1995). IL-10, a cytokine known to inhibit NF-κB activation (Wang *et al.*, 1995), has also been implicated in the mechanism by which PDE4 inhibitors suppress monocyte TNFα release (Kambayashi *et al.*, 1995).

The inhibitory potency difference between RP 73401 and rolipram on LPS-induced TNFα release was much greater (~70 fold) than was previously observed in studies on eosinophils and airways smooth muscle in which RP 73401 was only 3–14 fold more potent in suppressing functional responses (Souness *et al.*, 1995). The major discrepancy observed between the relative potencies of trequinsin and rolipram on esoinophil functional responses and PDE4 activity (Souness *et al.*, 1991) was not evident in monocytes. Trequinsin, which was approximately equipotent with (±)-rolipram against monocyte PDE4, was 6 fold less potent than rolipram in increasing cyclic AMP levels and only 3 fold less potent in reducing TNFα production. The suppression of TNFα release (and potentia-

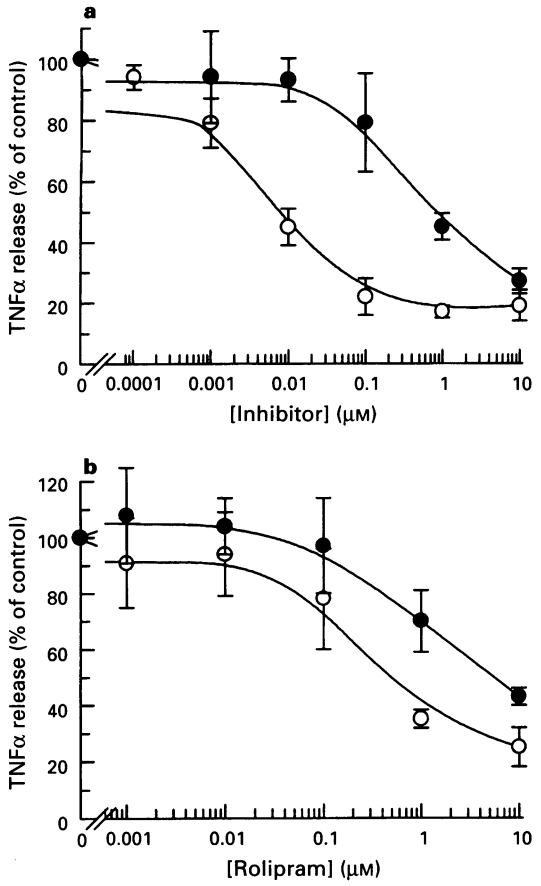

Figure 4 Inhibition of LPS (10 ng ml^{-1})-induced TNFα by (a) RP 73401 (●) and (±)-rolipram (○) and (b) **R**-(−)-rolipram (●) and **S**-(+)-rolipram (○). The results represent the mean±s.e.mean of 3–5 experiments. In samples treated with LPS alone, TNFα concentrations were 2952±432 pg ml^{-1}.

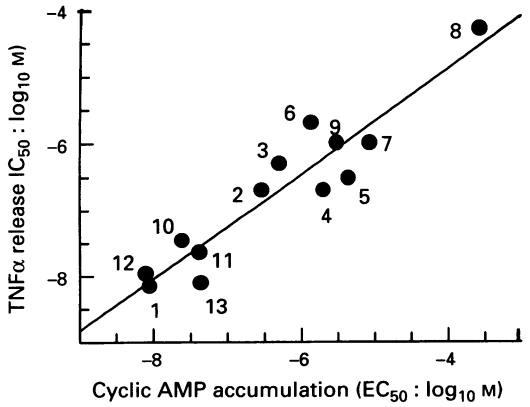

Figure 5 Inhibition of TNFα release as a function of cyclic AMP accumulation. Cyclic AMP data are expressed as EC_{50} values (log concn) and TNFα data is IC_{50} values (log concn). TNFα inhibition as a function cyclic AMP stimulation is highly significant ($r=0.94$, $P<0.01$, $n=13$). Each point is a mean of at least 3 determinations. The compound key is the same as that shown in the legend to Figure 3.

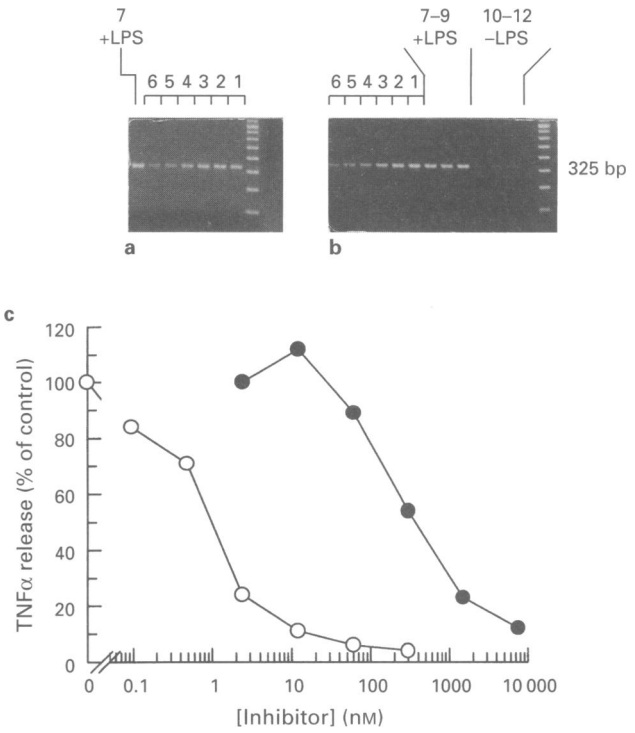

Figure 6 Suppression of TNFα mRNA expression by RP 73401 (○) and (±)-rolipram (●). The results are from a typical experiment. The insets (a, b) show the PCR products from ethidium bromide-stained gels. Inset A: [rolipram]: (1) 2.4 nM; (2) 12 nM; (3) 60 nM; (4) 300 nM; (5) 1500 nM; (6) 7500 nM; (7) LPS (1 ng ml^{-1}) alone. Inset B, [RP 73401]: (1) 0.096 nM; (2) 0.48 nM; (3) 2.4 nM; (4) 12 nM; (5) 60 nM; (6) 300 nM; (7–9) LPS alone; (10–12) no LPS.

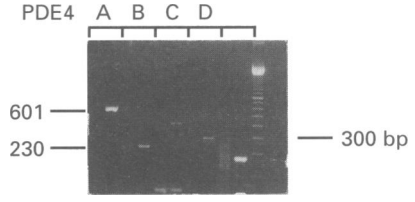

Figure 7 PDE4 subtype message amplification phenotyping in human monocytes. First strand cDNA synthesis and PCR amplification (55 cycles) (using primers recognizing unique sequences in human PDE4A-D of mRNA from human monocytes) were performed as described in the methods section.

tion of PGE_2-induced cyclic AMP accumulation) was better correlated with inhibition of monocyte PDE4 than displacement of [^{3}H]-rolipram binding to brain membranes. This again contrasts with the relationship between suppression of functional responses by PDE4 inhibitors in eosinophils (Souness & Scott, 1993) and airways smooth muscle (Harris *et al.*, 1989) which are very tightly correlated to their affinities for the HARBS.

Thus, the interactions of RP 73401 and rolipram with native PDE4 in monocytes and eosinophils appear to differ. One, possible explanation for this is that the complement of PDE4 subtypes in these two cell-types is distinct and one or other of the two inhibitors is subtype selective. Although our RT-PCR data demonstrate that eosinophils (type D) and monocytes (types A and B) express different PDE4 subtypes (at least at the mRNA level), rolipram does not exhibit sub-type selectivity (Bolger *et al.*, 1993) and because of its similar potencies against PDE4 preparations from a variety of sources (Ashton *et al.*, 1994; Souness *et al.*, 1995; data contained herein), this also appears to be the case with RP 73401. Thus, the sub-type hypothesis does not appear to offer a satisfactory explanation for the pharmacological anomalies with PDE inhibitors observed between monocytes and eosinophils.

Evidence has been documented suggesting that eosinophil PDE4 can adopt different conformations (Souness *et al.*, 1992; Souness & Scott, 1993). These putative conformational changes which are indicated by altered kinetic properties elicited by solubilization and V/GSH, are associated with an increase in enzyme sensitivity to the inhibitory actions of some compounds (rolipram, denbufylline) but not others (RP 73401, trequinsin, dipyridamole) (Souness *et al.*, 1992; Souness & Scott, 1993; Souness *et al.*, 1995). Close correlations between the inhibitor-induced cyclic AMP accumulation in intact eosinophils and inhibition of the solubilized or V/GSH-activated enzyme suggest that the native PDE4 in these cells exists in a form similar to that brought about by these treatments (Souness & Scott, 1993). How V/GSH acts on eosinophil PDE4 is uncertain – an electron transfer enzyme oxidation may be involved (Thompson *et al.*, 1991); alternatively, V/GSH may be mimicking the effects of phosphorylation. Eosinophils express PDE4D (Souness *et al.*, 1995), one splice variant of which (rPDE 3.3/PDE4D3) is known be phosphorylated and activated by PKA (Sette *et al.*, 1994). Activation of this splice variant of PDE4D is associated with increased sensitivity to the inhibitory actions of nitraquazone-like structures (RS-25344, RS-25344) but not trequinsin (Alvarez *et al.*, 1995). Monocytes express predominantly PDE4A and PDE4B (PDE4D-very weakly expressed) (Figure 7, see also Engels *et al.*, 1994; Verghese *et al.*, 1995). It is uncertain whether the monocyte PDE4(s) can adopt the conformational state(s) and the increased sensitivity to some inhibitors in a manner similar to the eosinophil enzyme, although V/GSH did increase slightly (40%) cytosolic hydrolytic activity. Certainly, the poor correlation between monocyte functional responses and displacement of [^{3}H]-rolipram binding indicates that, in contrast to eosinophils, the native monocyte PDE4 exists in a low-affinity rather than a high-affinity form. It should be noted, however, that [^{3}H]-rolipram binds with high affinity to both predominant monocyte PDE4 subtypes (Torphy *et al.*, 1992; McLaughlin *et al.*, 1993).

Support for the existence of distinct conformational states of PDE4 in different cells-types has recently emerged (Barnette *et al.*, 1995a,b). The molecular basis for the different hypothesized states of PDE4 was not elucidated. The proposal was based on pharmacological data on compounds exhibiting different relative potencies against human recombinant PDE4A and in displacing [^{3}H]-rolipram from its high-affinity receptor in brain cytosol (Barnette *et al.*, 1995a,b). The former activity was suggested to represent a low-affinity binding site on PDE4 (LPDE4) and the latter a high-affinity binding site (HPDE4). It was suggested that these different forms of PDE4 are non-interconvertible. In a range of studies with compounds which show great selectivity for high-affinity PDE4 (e.g. rolipram, denbufylline) or which exhibit slightly greater potency on low-affinity PDE4 or do not discriminate between the two sites (e.g. trequinsin, dipyridamole), it was concluded that suppression of certain inflammatory cell functions are associated, by and large, with actions on low-affinity PDE4, whereas the functional responses in other cells (e.g. parietal cells, airways smooth muscle) are more closely linked with actions on high-affinity PDE4 (Barnette *et al.*, 1995a,b). Based on this hypothesis, our data tempt speculation that the monocyte PDE4 represents a low-affinity PDE4 whereas the eosinophil enzyme is a high affinity PDE4 form.

If the above hypothesis is correct, it might be expected that, in contrast to eosinophils, little or no rolipram stereoselectivity would be observed on functional responses in monocytes; however, approximately 5 fold stereoselectivity was observed in rolipram's suppression of TNFα release and potentiation of PGE_2-induced cyclic AMP accumulation. Previously, 10 fold stereoselectivity of rolipram was reported for LPS-induced TNFα release from human mononuclear cells (Semmler *et al.*, 1993). This compares with 10 fold rolipram stereoselectivity in elevating eosinophil cyclic AMP levels (Souness & Scott, 1993). Thus, it may be too simplistic to envisage a situation in which PDE4 can exist exclusively in a high-affinity form in some cells and a low-affinity form in others. Perhaps, in cells, such as monocytes, where multiple subtypes are expressed, PDE4 may be present in both high-affinity and low-affinity forms and the proportion of enzyme(s) existing in these different states, as dictated by intracellular conditions, would determine whether functional responses to PDE inhibitors are better correlated with PDE4 inhibition or displacement of rolipram binding.

Although potency differences between RP 73401 and rolipram may provide insight into the nature of native PDE4, it is important to remember that factors other than intrinsic PDE4 inhibitory activity will influence the absolute potencies of inhibitors in eliciting functional responses in intact cells. For example, whereas the IC_{50} values of RP 73401 against PDE4 preparations from a variety of sources are very similar (Souness *et al.*, 1995; data contained herein), its potencies in eliciting whole-cell responses vary – RP 73401 is almost 40 fold more potent in inhibiting LPS-induced TNFα release from monocytes than LTB_4-stimulated MBP release from guinea-pig eosinophils (Souness *et al.*, 1995). This may be due to differences between cell-types in the proportion of total PDE4 activity required to be inhibited for elevation of intracellular cyclic AMP to occur, which, in turn, will be dependent upon the relative intracellular rates of cyclic AMP synthesis and hydrolysis (turnover). Unequal uptake of PDE4 inhibitors may also be a factor to consider when comparing their potencies on the functional responses in different cells and tissues. Unfortunately, little documented information is available to assess its importance.

In conclusion RP 73401 is a very potent inhibitor of TNFα release from human monocytes suggesting that it may have therapeutic potential in the many pathological conditions associated with over-production of this pro-inflammatory cytokine. The data suggest that the native PDE4s in monocytes and eosinophils may exist in different states. RP 73401 exhibits similar potencies against these two hypothesized states whereas rolipram is much more potent against a high-affinity form of PDE4, proposed to exist in eosinophils, than a low-affinity form, proposed to be expressed in monocytes. Thus, the potency difference between the two compounds is much greater in monocytes than in eosinophils.

References

ALVAREZ, R., SETTE, C., YANG, D., EGLEN, R.M., WILHELM, R., SHELTON, E.R. & CONTI, M. (1995). Activation and selective inhibition of a cyclic AMP-specific phosphodiesterase, PDE-4D3. *Mol. Pharmacol.*, **48,** 616–622.

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-meth oxybenzamides and analogues. *J. Med. Chem.*, **37,** 1696–1703.

BARNETTE, M.S., GROUS, M., CIESLINSKI, L.B., BURMAN, M., CHRISTENSEN, S.B. & TROPHY, T.J. (1995a). Inhibitors of phosphodiesterase IV (PDE IV) increase acid secretion in rabbit isolated gastric glands: Correlation between function and interaction with a high-affinity rolipram binding site. *J. Pharmacol. Exp. Ther.*, **273,** 1396–1402.

BARNETTE, M.S., MANNING, C.D., CIESLINSKI, L.B., BURMAN, M., CHRISTENSEN, S.B. & TORPHY, T.J. (1995b). The ability of phosphodiesterase IV inhibitors to suppress superoxide production in guinea-pig eosinophils is correlated with inhibition of phosphodiesterase4 catalytic activity. *J. Pharmacol. Exp. Ther.*, **273,** 674–679.

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.

BOLGER, G. (1994). Molecular biology of the cyclic AMP-specific cyclic nucleotide phosphodiesterases: A diverse family of regulatory enzymes. *Cell. Signalling*, **6,** 851–859.

BOLGER, G., MICHAELI, T., MARTINS, T., ST JOHN, T., STEINER, B., RODGERS, L., RIGGS, M., WIGLER, M. & FERGUSON, K. (1993). A family of human phosphodiesterases homologous to the dunce learning and memory gene product of *Drosophila melanogaster* are potential targets for anti-depressant drugs. *Mol. Cell. Biol.*, **13,** 6558–6571.

ENGELS, P., FITCHEL, K. & LUBBERT, H. (1994). Expression and regulation of human and rat phosphodiesterase type IV isogenes. *FEBS Lett.*, **350,** 291–295.

FAUCI, A.S., SCHNITTMAN, S.M., POLI, G, KOENIG, S. & PANTALEO, G. (1991). Immunopathogenic mechanisms in human immunodeficiency virus (HIV) infection. *Ann. Intern. Med.*, **114,** 678–693.

FAZELY, F., DEZUBE, B.J., ALLEN-RYAN, J., PARDEE, A.B. & RUPRECHT, R.M. (1991). Pentoxifylline (Trental) decreases the replication of the human immunodeficiency virus type 1 in human peripheral blood mononuclear cells and cultured T-cells. *Blood*, **77,** 1653–1656.

FEUERSTEIN, G.Z., LIU, T. & BARONE, F.C. (1994). Cytokines, inflammation and brain injury: Role of the tumour necrosis factor-α. *Cerebrovasc. Brain. Metab. Rev.*, **6,** 341–360.

GENAIN, C.P., ROBERTS, T., DAVIS, R.L., NGUYEN, M.-H., UCCELLI, A., FAULDS, D., LI, Y., HEDGPETH, J. & HAUSER, S.L. (1995). Prevention of autoimmune demyelination in non-human primates by a cAMP-specific phosphodiesterase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.*, **92,** 3601–3605.

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.

HINSHAW, L.B., EMERSON, T.E., TAYLOR, F.B., CHANG, A.C.K., DUERR, M., PEER, G.T., FLOURNOY, D.J., WHITE, G.L., KOSANSKE, S.D., MURRAY, C.K., XU, R., PASEY, R.B. & FOURNEL, M.A. (1992). Lethal Staphylococcus aureus-induced shock in primates: Prevention of death with anti-TNF antibody. *J. Trauma*, **33,** 568–573.

ISHIKAWA, Y., MUKAIDA, N., KUNO, K., RICE, N., OKAMOTO, S.-I. & MATSUSHIMA, K. (1995) Establishment of lipopolysaccharide-dependent nuclear factor κB activation in cell-free system. *J. Biol. Chem.*, **270,** 4158–4164.

KAMBAYASHI, T., JACOB, C.O., ZHOU, D., MAZUREK, N., FONG, M. & STRASSMANN, G. (1995). Cyclic nucleotide phosphodiesterase type IV participates in the regulation of IL-10 and the subsequent inhibition of TNF-α and IL-6 release by endotoxin-stimulated macrophages. *J. Immunol.*, **155,** 4909–4916.

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.

MURRAY, K.J., ENGLAND, P.J., HALLAM, T.J., MAGUIRE, J., MOORES, K., REEVES, M.L., SIMPSON, A.W.M. & RINK, T.J. (1990). The effects of siguazodan, a selective phosphodiesterase inhibitor, on human platelet function. *Br. J. Pharmacol.*, **99,** 612–616.

NEWELL, C.L., DEISSEROTH, A.B. & LOPEZ-BERESTEIN, G. (1994). Interaction of nuclear proteins with an AP-1/CRE-like promoter sequence in the human TNFα gene. *J. Leukoc. Biol.*, **56,** 27–35.

PALFREYMAN, M.N. & SOUNESS, J.E. (1996) Phosphodiesterase type IV inhibitors. *Prog. Med. Chem.*, **33,** 1–52.

PRABHAKAR, U., LIPSHUTZ, D., O'LEARY BARTUS, J., SLIVJAK, M.J., SMITH III, E.F., LEE, J.C. & ESSER, K.M. (1994). Characterization of cAMP-dependent inhibition of LPS-induced TNFα production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor. *Int. J. Immunopharmacol.*, **16,** 805–816.

SATRIANO, J. & SCHLONDORFF, D. (1994). Activation and attenuation of transcription factor NF-κB in mouse glomerular mesangial cells in reponse to tumor necrosis factor-α, immunoglobulin G, and adenosine 3':5'-cyclic monophosphate: Evidence for involvement of oxygen species. *J. Clin. Invest.*, **94,** 1629–1636.

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

SCHMEICHEN, R., SCHNEIDE, H.H. & WACHTEL, H. (1990). Close correlation between behavioral responses and binding in vivo for inhibitors of the rolipram-sensitive phosphodiesterase. *Psychopharmacology*, **102,** 17–20.

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.

SEKUT, L., YARNALL, D., STIMPSON, S.A., NOEL, L.S., BATEMANFITE, R., CLARK, R.L., BRACKEEN, M.F., MENIUS, L.A. & CONNOLLY, K.M. (1995). Anti-inflammatory activity of phosphodiesterase (PDE) IV inhibitors in acute and chronic models of inflammation. *Clin. Exp. Immunol.*, **100,** 126–132.

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 activiated by cAMP-dependent protein kinase. *J. Biol. Chem.*, **269,** 18271–18274.

SOMMER, N., LOSCHMANN, P.-A., NORTHOFF, G.H., WELLER, M., STEINBRECHER, A., STEINBACH, J.P., LICHTENFELS, R., MEYERMANN, R., RIETHMULLER, A., FONTANA, A., DICHGANS, J. & MARTIN, R. (1995). The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nature Med.*, **1,** 244–248.

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

SOUNESS, J.E., MASLEN, C., WEBBER, S., FOSTER, M., RAEBURN, D., PALFREYMANN, M.N., ASHTON, M.J. & KARLSSON, J.-A. (1995). Suppression of eosinophil function by RP 73401, a potent and selective inhibitor of cyclic AMP-specific phosphodiesterase: comparison with rolipram. *Br. J. Pharmacol.*, **114,** 39–46.

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.

THOMPSON, W.J., ROSS, C.P., PLEDGER, W.J., STRADA, S.J., BANNEWR, R.L. & HERSH, E.M. (1976). Cyclic adenosine 3':5'-monophosphate phosphodiesterase. Distinct forms in human lymphocytes and monocytes. *J. Biol. Chem.*, **251,** 4922–4929.

THOMPSON, W.J., TAN, B.H. & STRADA, S.J. (1991). Activation of rabbit liver high-affinity cAMP (Type IV) phosphodiesterase by vanadyl-glutathione complex. *J. Biol. Chem.*, **266,** 17011–17019.

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. (1994). Inhibitors of phosphodiesterase isozymes: new therapeutic possibilities for asthma. In *Bronchitis V.* ed. Postma, D. & Gerritson, J. pp. 303–316. Assen: Van Gorcum & Co.

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

TRACEY, K.J. & CERAMI, A. (1993). Tumour necrosis factor, other cytokines and disease. *Annu. Rev. Cell Biol.*, **9,** 317–343.

VERGHESE, M.W., MCCONNELL, R.T., LENHARD, J.M., HAMACHER, L. & JIN, S.-L.C. (1995). Regulation of distinct cyclic AMP-specific phosphodiesterase (phosphodiesterase type 4) isozymes in human monocytic cells. *Mol. Pharmacol.*, **47,** 1164–1171.

WAAGE, A., SORENSEN, M. & STORDAL, B. (1990). Differential effect of oxpentifylline on tumour necrosis factor and interleukin-6 production. *Lancet*, **335,** 543.

WANG, P., WU, P., SIEGEL, M.I., EGAN, R.W. & BILLAH, M.M. (1995). Interleukin (IL)-10 inhibits factor κB (NFκB) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *J. Biol. Chem.*, **270,** 9558–9563.

WHITE, J.R., TORPHY, T.J., CHRISTENSEN, S.B., LEE, J.A. & MONG, S. (1990). Purification and characterization of the rolipram sensitive, low Km phosphodiesterase from human monocytes. *FASEB J.*, **4,** A1987.

WILLIAMS, R.O., FELDMAN, M. & MAINI, R.N. (1992). Anti-tumour necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 9784–9788.

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