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Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4$^+$ and CD8$^+$ T-lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2

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1 The cyclic AMP phosphodiesterases (PDE) expressed by CD4$^+$ and CD8$^+$ T-lymphocytes purified from the peripheral blood of normal adult subjects were identified and characterized, and their role in modulating proliferation and the biosynthesis of interleukin (IL)-2 and interferon (IFN)-γ evaluated.

2 In lysates prepared from both subsets, SK&F 95654 (PDE3 inhibitor) and rolipram (PDE4 inhibitor) suppressed cyclic AMP hydrolysis indicating the presence of PDE3 and PDE4 isoenzymes in these cells. Differential centrifugation and subsequent inhibitor and kinetic studies revealed that the particulate fraction contained, predominantly, a PDE3 isoenzyme. In contrast, the soluble fraction contained a PDE4 ($\sim$65% of total activity) and, in addition, a novel enzyme that had the kinetic characteristics of the recently identified PDE7.

3 Reverse transcription-polymerase chain reaction (RT-PCR) studies with primer pairs designed to recognise unique sequences in the human PDE4 and PDE7 genes amplified cDNA fragments that corresponded to the predicted sizes of HSPDE4A, HSPDE4B, HSPDE54D and HSPDE7. No message was detected for HSPDE4C after 35 cycles of amplification.

4 Functionally, rolipram inhibited phytohaemagglutinin- (PHA) and anti-CD3-induced proliferation of CD4$^+$ and CD8$^+$ T-lymphocytes, and the elaboration of IL-2, which was associated with a three to four fold increase in cyclic AMP mass. In all experiments, however, rolipram was approximately 60 fold more potent at suppressing IL-2 synthesis than at inhibiting mitogenesis. In contrast, SK&F 95654 failed to suppress proliferation and cytokine generation, and did not elevate the cyclic AMP content in T-cells. Although inactive alone, SK&F 95654 potentiated the ability of rolipram to suppress PHA- and anti-CD3-induced T-cell proliferation, and PHA-induced IL-2 release.

5 When a combination of phorbol myristate acetate (PMA) and ionomycin were used as a co-mitogen, rolipram did not affect proliferation but, paradoxically, suppressed IL-2 release indicating that cyclic AMP can inhibit mitogenesis by acting at, or proximal to, the level of inositol phospholipid hydrolysis.

6 Collectively, these data suggest that PDE3 and PDE4 isoenzymes regulate the cyclic AMP content, IL-2 biosynthesis and proliferation in human CD4$^+$ and CD8$^+$ T-lymphocytes. However, the ability of rolipram to suppress markedly mitogen-induced IL-2 generation without affecting T-cell proliferation suggests that growth and division of T-lymphocytes may be governed by mediators in addition to IL-2. Finally, T-cells have the potential to express PDE7, although elucidating the functional role of this enzyme must await the development of selective inhibitors.

Keywords: CD4$^+$ T-lymphocytes; CD8$^+$ T-lymphocytes; phosphodiesterase inhibitors; proliferation; interleukin-2 generation; phosphodiesterase 3; phosphodiesterase 4; phosphodiesterase 7

Introduction

Although glucocorticosteroids are considered the most effective agents currently available for the treatment of asthmatic inflammation, they are not without adverse effects which has prompted a search for new compounds with enhanced selectivity and improved side-effect profiles. From a theoretical perspective, one group of potentially steroid-sparing drugs that may exhibit powerful anti-inflammatory and immunomodulatory activity are inhibitors of the cyclic nucleotide phosphodiesterase (PDE) isoenzymes (E.C. 3.1.4.17, E.C. 3.1.4.35) (Torphy & Undem, 1991; Giembycz, 1992; Dent & Giembycz, 1995). Seven families have thus far been identified (see Beavo *et al.*, 1994) which encode theoretically more than 34 structurally distinct proteins that are responsible for the highly regulated and co-ordinated inactivation of the second messenger purine nucleotides, adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Beavo & Reifsnyder, 1990; Giembycz & Kelly, 1994; Loughney & Ferguson, 1994).

With respect to treating asthmatic inflammation, the PDE4 isoenzyme family has been identified as a viable target amenable to therapeutic intervention with selective inhibitors. The rationale for developing PDE4 inhibitors is based upon the finding that PDE4 is the predominant cyclic AMP-metabolising enzyme in essentially all pro-inflammatory and immune cells implicated in the pathogenesis of asthma (Torphy & Undem, 1991; Giembycz, 1992; Dent & Giembycz, 1995). Moreover, inhibition of PDE4 suppresses an array of functional indices of cell activation that are considered pro-inflammatory (Torphy & Undem, 1991; Giembycz, 1992; Dent & Giembycz, 1995). An additional level of complexity is provided by the fact that PDE4 is a generic term used to describe a large group of similar but, nevertheless, structurally, functionally and immunologically distinct proteins which are differentially expressed between cells and tissues (Davis *et al.*, 1989; Conti *et al.*, 1992; Welsh *et al.*, 1992). Indeed, four mammalian genes (PDE4A-D) have been identified that can theoretically encode more than 16 proteins which arise from alternative mRNA splicing and/or from transcription of these genes at multiple promoter regions (Davis *et al.*, 1989; Conti *et al.*, 1992; Welsh *et al.*, 1992; Bolger *et al.*, 1993; Beavo *et al.*,

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1994; Giembycz & Kelly, 1994; Loughney & Ferguson, 1994; Monaco *et al.*, 1994). This marked heterogeneity within the PDE4 family may allow for the development of, so-called, subfamily-selective inhibitors which could enable more selective targeting and discrete functional manipulation of those cells which express the PDE4 isoenzyme of interest.

Of those cells that have been implicated in asthma pathogenesis, T-lymphocytes play a central role and are believed to orchestrate the chronic eosinophilic inflammation that characterizes this disease (see Corrigan & Kay, 1992). Previous studies have established that a number of cyclic AMP-elevating drugs and lipophilic cyclic AMP analogues suppress various functional indices of T-cell activation including exocytosis (Takayama *et al.*, 1988), proliferation (Estes *et al.*, 1971; Goodwin *et al.*, 1977; Lingk *et al.*, 1990; Krause & Deutsch, 1991), cytokine (interferon-γ (IFNγ), interleukin-2 (IL-2) and IL-4) induction (Averill *et al.*, 1988; Wacholz *et al.*, 1991; Anastassoiu *et al.*, 1992; Ganea & Sun, 1993; Snijdewint *et al.*, 1993), and the expression of CD7 (Rincón *et al.*, 1992) and high and low affinity IL-2 receptors (Rincón *et al.*, 1988; Krause & Deutsch, 1991). In spite of these data, there is a paucity of literature relating to the complement and characteristics of PDEs in T-lymphocytes and the biochemical and functional effects which occur following exposure of these cells to isoenzyme-selective PDE inhibitors. This is, perhaps, surprising since PDEs in T-lymphocytes were originally described more than 20 years ago (Thompson *et al.*, 1976; Takemoto *et al.*, 1978). Nevertheless, Averill *et al.* (1988) have shown that a high concentration (100 μM) of Ro 20-1724 suppressed the induction of IL-2 in an enriched population of T-lymphocytes in response to phytohaemagglutinin (PHA), and equivalent data have been published with non-selective PDE inhibitors including 3-isobutyl-1-methylxanthine (IMBX), pentoxifylline, theophylline and caffeine (Mary *et al.*, 1987; Rott *et al.*, 1993; Snijewint *et al.*, 1993). More recently, Robiscek *et al.* (1991) documented that a PDE3 (CI-930) and PDE4 (Ro 20-1724) inhibitor attenuated mitogen-induced [^{3}H]-thymidine incorporation into a mixed population of T-lymphocytes and provided evidence that these cells express representatives of the PDE3 and PDE4 isoenzyme families (Robiscek *et al.*, 1989; 1991).

In this paper, we present results of detailed experiments designed to identify and characterize the PDEs expressed in highly purified human peripheral blood CD4$^+$ and CD8$^+$ T-lymphocytes by use of a combination of biochemical and molecular techniques. In addition, the role of these enzymes in regulating cyclic nucleotide levels in intact cells, and the effects of selective PDE inhibitors on mitogen-induced proliferation and on the biosynthesis of IL-2 and IFNγ are described. A preliminary account of some of these data was presented to the American Academy of Allergy and Immunology (Giembycz *et al.*, 1994).

Methods

Harvesting and purification of human T-lymphocytes

Blood ($\sim$100 ml) was taken from normal healthy volunteers taking no medication by ante-cubital venepuncture into sterile, preservative-free heparin (1% v/v; 5000 u ml^{-1}). Granulocytes were then isolated by centrifugation essentially as described by Böyum (1968). The resulting leukocyte-rich plasma was mixed with an equal volume of RPMI-1640 and aliquots (30 ml) were layered onto 20 ml of Ficoll-Hypaque in 50 ml sterile polypropylene conical tubes (Falcon, Becton-Dickinson, Cowley). After centrifugation (1200 $\times g$, 20 min, 20°C) the peripheral blood mononuclear cells (PBMCs) were removed from the plasma/Ficoll-Hypaque interface by gentle suction, washed twice in RPMI 1640 and resuspended as necessary.

Isolation of CD4$^+$ and CD8$^+$ T-lymphocytes

T-Lymphocyte subsets were isolated from PBMCs by magnetisable beads coated with murine anti-CD4 or anti-CD8 monoclonal antibodies (Dynabeads, Dynal, New Ferry) according to the manufacturer's instructions. Briefly, 150×10^6 peripheral blood mononuclear cells were suspended in 2 ml RPMI-1640 medium containing 2% v/v heat-inactivated FCS and placed on ice. Anti-CD4$^+$ or anti-CD8$^+$ (75×10^6 each) Dynabeads were then added in 175 μl of the same medium. After gentle agitation (20 min; 4°C) the T-lymphocytes were isolated in a magnetic field and washed three times in ice-cold medium to remove platelets. The purity of the T-cell subsets, verified by flow cytometry, was never less than 96%.

Measurement of T-lymphocyte proliferation

Purified CD4$^+$ and CD8$^+$ T-lymphocytes were resuspended in supplemented RPMI 1640 at 4×10^6 cells ml^{-1}. One hundred microlitres of these cell suspensions were added in triplicate to 20 μl aliquots of serially-diluted drug solutions (rolipram, SK&F 95654) or their respective vehicle in sterile 96-well round-bottomed culture plates (Cel-Cult, Sterilin, Hounslow) containing PHA (final concentration 5 μg ml^{-1}), anti-CD3 (final concentration 500 ng ml^{-1}), PMA/ionomycin (final concentration 1 μM each) or medium to a final volume of 200 μl. Culture plates were incubated (37°C, 72 h) in a humidified atmosphere containing 5% CO_2 and cell proliferation subsequently assessed by measuring the incorporation of thymidine. Briefly, sterile [^{3}H]-thymidine (24.6 kBq/well in 10 μl) was added to cell culture wells for the last 8 h of the 72 h incubation period and cells were then harvested on to glass-fibre filter paper by vacuum filtration and incorporated radioactivity counted at an efficiency of $\sim$60%.

Treatment of T-lymphocytes for IL-2 and IFNγ generation

Aliquots (2×10^6 cells in 1 ml) of purified CD4$^+$ and CD8$^+$ T-lymphocytes were cultured in sterile 5 ml polystyrene tissue culture tubes (Cel-Cult, Sterilin, Hounslow) for 16 h at 37°C under a humidified atmosphere containing 5% CO_2 in the absence and presence of mitogens (PHA – 5 μg ml^{-1}, anti-CD3 – 500 ng ml^{-1}, PMA/ionomycin – 1 μM each) and PDE inhibitors (rolipram – 1 nM to 10 μM, SK&F 95654 – 1 nM to 10 μM) as indicated in the text. Supernatants were harvested from the cultures and stored at −80°C pending measurement of IL-2 and IFNγ.

Measurement of IL-2 and IFNγ

The amount of IL-2 and IFNγ in culture supernatants was measured by specific ELISAs calibrated with human recombinant IL-2 (0–2000 pg ml^{-1}) and IFNγ (0–6400 pg ml^{-1}), respectively. The sensitivity of these assays, defined as the concentration of cytokine required to produce an optical density 2 standard deviations above the mean optical density of 20 zero standard replicates, is 6 pg ml^{-1} for IL-2 and 10 pg ml^{-1} for IFNγ. Intra- and inter-assay coefficients of variation did not exceed 7 and 9.2%, respectively, for both ELISAs. The culture supernatant was routinely diluted such that the concentration of cytokine measured was within the limits of the appropriate standard curve.

Measurement of cyclic AMP

Purified T-cell subsets were resuspended at 15×10^6 ml^{-1} in buffer A (in mM: HEPES 10 - pH 7.4, NaCl 124, KCl 4, NaH_2PO_4 0.64, K_2HPO_4 0.66, $NaHCO_3$ 5.2, $CaCl_2$ 1.6, glucose 5.6, $MgCl_2$ 1) and stored on ice until required. Assays, performed in duplicate, were conducted at 37°C in a shaking water bath in a total volume of 300 μl and were initiated by the addition of 30 μl of T-cell suspension (1×10^6 cells) to 270 μl pre-warmed buffer A. To examine the effect of PDE inhibitors on lymphocyte cyclic AMP content, compounds (30 μl) were routinely added to the cell suspensions at the concentrations indicated in the text and relevant figures for 10 min. Reactions

were terminated by the addition of 300 μl ice-cold trichloroacetic acid (TCA; 1 M), centrifuged to precipitate particulate material and the supernatants neutralised. Aliquots (500 μl) of the neutralised extracts were acetylated by the consecutive addition of triethylamine (20 μl) and acetic anhydride (10 μl), and cyclic AMP mass was measured immediately by RIA. Briefly, to 200 μl of acetylated sample, were added 50 μl of adenosine-3',5'-monophospho-2-*O*-succinyl-3-[^{125}I]-iodotyrosine methyl ester (approximately 2000–3000 d.p.m.) in 0.2% BSA and 100 μl of anti-cyclic AMP antibody in 0.2% BSA. After vortex-mixing, samples were incubated overnight at 4°C and free and antibody-bound cyclic AMP was separated by charcoal precipitation with ice-cold potassium phosphate buffer (100 mM in 0.2% BSA–pH 7.4) and quantified by γ-counting. The detection limit and sensitivity (IC_{50}) of this assay are 10 and 145 fmol cyclic AMP, respectively.

Measurement of cyclic nucleotide PDE activity

PDE activity was measured by a modification (Schwartz & Passonneau, 1974) of the method of Thompson and Appleman (1971) as described by Dent *et al.* (1994). The reaction is based upon the breakdown of [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP by PDE to the corresponding labelled nucleoside 5'-monophosphate which is subsequently dephosphorylated by alkaline phosphatase. T-cells were lysed osmotically in ice-cold buffer B (20 mM TEA - pH 8, 1 mM EDTA) supplemented with the proteinase inhibitors benzamidine (2 mM), leupeptin (50 μM), PMSF (100 μM), bacitracin (100 μg ml^{-1}) and soybean trypsin inhibitor (20 μg ml^{-1}) and either used immediately or centrifuged at 145 000 *g* for 30 min at 4°C to provide soluble and membrane fractions. Assays were performed in duplicate at 37°C and initiated by the addition of 30 μl enzyme (cell lysate, or membrane-bound or solubilised fraction) to 270 μl of a reaction cocktail containing (final concentration) 20 mM TEA (pH 8.0), 5 mM DTT, 500 μg ml^{-1} BSA, 5 mM magnesium acetate, 0.25 unit alkaline phosphatase, 1 mM EGTA, 1 μM cyclic NMP (supplemented with *ca.* 250 000 d.p.m. of [8-^{3}H] cyclic NMP and *ca.* 5 000 d.p.m. [8-^{14}C]-adenosine to estimate recovery) and the PDE inhibitor under evaluation or its vehicle. The reaction was terminated by the addition of 1 ml of a mixture of Dowex AG 1-×8: methanol: water (1:2:1), vortex-mixed and placed in an ice bath until the end of the assay. Samples were then further vortex-mixed for 30 min before being centrifuged at 12 000 *g* for 5 min at 4°C. The radioactivity in 700 μl aliquots of the resulting supernatants was determined by liquid scintillation counting in 2 ml ACS II scintillant (Amersham International, Buckinghamshire) at a counting efficiency of approximately 60%. PDE activity is expressed as the formation of nucleoside 5' monophosphate from cyclic NMP per minute per 10^6 cell equivalents (i.e. the lysate derived from 10^6 cells) at 37°C after correction for the recovery (approximately 65–85%) of [8-^{14}C]-adenosine.

Classification of cyclic nucleotide PDEs

Cyclic nucleotide PDEs are classified according to the new nomenclature outlined in Beavo *et al.* (1994). Thus, HSPDEs 1 to 7, refer to the Ca^{2+}/calmodulin-dependent, cyclic GMP-stimulated, cyclic GMP-inhibited, cyclic AMP-specific, cyclic GMP-specific, photoreceptor and rolipram-insensitive, cyclic AMP-specific PDE families respectively, where the prefix, HS, refers to the species, *Homo sapiens*.

Determination of kinetic constants

Values of K_m and V_{max} were determined by varying the amount of unlabelled cyclic AMP in the reaction cocktail in the presence of a fixed concentration of radiolabelled cyclic nucleotide tracer. Appropriate corrections were made for the changes in specific activity of the substrate. Hofstee plots were constructed and analysed by linear regression by the method of least squares. Where kinetic analysis of cyclic AMP hydrolysis yielded curvilinear plots, the data were resolved into two components by the method of Spears *et al.* (1971) making the assumption that both activities conformed to Michealis-Menten kinetic behaviour.

Detection of HSPDE4A, HSPDE4B, HSPDE4D and HSPDE7 mRNA by RT-PCR

Total RNA was extracted from approximately 5×10^6 CD4$^+$ and CD8$^+$ T-lymphocytes as detailed in Chomzcynski and Sacchi (1987). One microgram of RNA was reverse transcribed by AMV Reverse Transcriptase (Promega, Southampton) according to the manufacturer's instructions and RT-generated cDNAs encoding the PDE4 and PDE7 genes were amplified by PCR with specific primers designed from their primary sequences (Bolger *et al.*, 1993; Michaeli *et al.*, 1993) deposited with the GenBank data base (Table 1). To confirm the integrity of T-cell RNA and equal loading of sample, reverse transcription-polymerase chain reaction (RT-PCR) analysis of the GAPDH gene was routinely performed with primers synthesized from sequences described in Maier *et al.* (1990). PCR amplification was conducted in a reaction volume of 25 μl by a Hybaid OmniGene thermal cycler (Hybaid, Teddington, Middlesex) and 0.5 u *Taq* polymerase set for 25 (GAPDH) or 35 (all PDEs) cycles under the conditions outlined in Table 1. PCR products were subsequently size-fractionated on 2% agarose gels, stained with ethidium bromide and visualised under u.v. light.

To control for possible genomic contamination of DNA samples, PCR was also performed on 100 ng genomic DNA, and test sample RNA was processed in parallel with the reversed transcribed sample in the absence of reverse transcriptase. To guard against contamination by PCR products, water blanks were subjected to PCR in parallel with test samples.

Drugs and analytical reagents

The following reagents were obtained from the Sigma Chemical Company (Poole, Dorset): benzamidine, bacitracin, leupeptin, soy bean trypsin inhibitor (STI), phenylmethylsulphonyl fluoride (PMSF), PHA, alkaline phosphatase (P-2277), EHNA (*erythro*-9-(2-hydroxyl-3-nonyl)adenine), triethylamine, acetic anhydride, ethidium bromide, cyclic AMP, cyclic GMP, anti-cyclic AMP antibody, calmodulin and Dowex AG (1 × 8-400 chloride form). Ficoll-Hypaque was purchased from LKB/Pharmacia (Milton Keynes, Buckinghamshire), *Taq* polymerase was from Bioline (Finchley, London) and foetal calf serum (FCS), L-glutamine and RPMI 1640 were from Gibco (Flow Laboratories, Rickmansworth, Hertfordshire). Anti-CD3 monoclonal antibody (UCHT-1) was kindly provided by Dr Doreen Cantrell (University College Hospital, London). [2,8-^{3}H]-cyclic AMP (36 Ci mmol^{-1}), [8-^{3}H]-cyclic GMP (24 Ci mmol^{-1}), [8-^{14}C]-adenosine (55 Ci mmol^{-1}), adenosine-3',5'-cyclic monophospho-2'-*O*-succinyl 3-[^{125}I]-iodotyrosylmethyl ester (~2000 Ci mmol^{-1}) and [^{3}H]-thymidine (29 Ci mmol^{-1}) were supplied by Amersham International (Little Chalfont, Buckinghamshire). Rolipram (**RS**-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone), SK&F 95654 (**RS**-4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyridin-1-yl)phenyl]5-methyl-3(2H) pyradazinone) and zaprinast (2-*O*-propoxyphenyl-8-azapurin-6-one) were kindly donated by Schering GmbH, (Berlin, Germany), SmithKline-Beecham (Welwyn Garden City, Hertfordshire) and Rhone-Poulenc Rorer Ltd (Dagenham, Essex), respectively. IL-2 (Quantikine) and IFNγ (Intertest-γ) enzyme linked immunosorbent assay (ELISA) kits were from Genzyme (Cambridge, Massachusetts, U.S.A.) and R & D Systems (Minneapolis, Minnesota, U.S.A.), respectively, and primer pairs designed against unique sequences of the PDE4, PDE7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were custom synthesized by R & D systems Europe Ltd (Abingdon). All other reagents were obtained from British Drug Houses (Poole, Dorset).

All drug dilutions for T-cell culture studies were made in RPMI 1640 supplemented with L-glutamine (2 mM) and 5% v/v heat-inactivated FCS. PHA was prepared at a stock concentration of 100 mg ml^{-1}, filtered (0.22 μm pore size) and stored at −20°C in aliquots. Anti-CD3 was stored at −80°C as a 1 mg ml^{-1} solution and, when required, added to T-cell cultures in liquid phase. Rolipram, SK&F 95654, phorbol myristate acetate (PMA) and ionomycin were made up as stock solutions of 100 mM in dimethyl sulphoxide (DMSO), filtered sterile and diluted to the desired working concentration as indicated.

Analysis of data and statistics

Where appropriate, data were analysed by least-squares, non-linear iterative regression with the 'PRISM' curve-fitting program (GraphPad Software, San Diego, California, U.S.A.). Values in the text, and data in figures and tables represent the mean ± s.e.mean of n independent determinations. For the proliferation studies, the geometric means of the triplicate counts were calculated and expressed as percentage inhibition of incorporated radioactivity measured in the absence of PDE inhibitors. IC_{50} values refer to the molar concentration of drug that was required to inhibit cyclic nucleotide hydrolysis, proliferation and cytokine generation by 50% whereas EC_{50} refers to the molar concentration of drug that produced 50% of the maximal effect elicited by that drug. Where statistical evaluation was required, data were analysed parametrically by Student's unpaired t test or, where appropriate, analysis of variance followed by a Dunnett's multiple comparison test. The null hypothesis was rejected when $P<0.05$.

Results

Complement of cyclic nucleotide PDEs expressed in $CD4^+$ and $CD8^+$ T-lymphocytes

At a substrate concentration of 1 μM, human peripheral blood $CD4^+$ and $CD8^+$ T-lymphocyte lysates hydrolysed cyclic purine nucleotides with a preference for cyclic AMP (1.27 ± 0.21 and 1.16 ± 0.11 pmol $min^{-1}/10^6$ cell eq., respectively, $n=4$) over cyclic GMP (0.27 ± 0.03 and 0.31 ± 0.04 pmol $min^{-1}/10^6$ cell eq., respectively, $n=4$). Cyclic AMP PDE activity was partially suppressed by rolipram (10 μM), cyclic GMP (10 μM) and SK&F 95654 (10 μM), but was unaffected by calmodulin (50 units plus 2 mM Ca^{2+}) and EHNA, indicating that both T-lymphocyte subsets express representatives of the PDE3 and PDE4 isoenzyme families but no detectable PDE1 or PDE2 (Figure 1). The relatively low level of cyclic GMP PDE activity in $CD4^+$ and $CD8^+$ T-cell lysates was significantly (>80%) inhibited by zaprinast (10 μM) indicating the expression of a HSPDE5 isoenzyme (data not shown).

Differential centrifugation (145,000 × g, 30 min, 4°C) of T-cell lysates revealed that PDE3 was predominantly, if not exclusively, membrane-associated whereas PDE4 was apparently soluble in nature (Figure 2). Indeed, in the particulate fraction SK&F 95654 suppressed cyclic AMP hydrolysis in a concentration-dependent manner (IC_{50}: 0.71 ± 0.02 and 0.63 ± 0.04 μM for $CD4^+$ and $CD8^+$ T-lymphocytes respectively, $P>0.05$) under conditions where rolipram (10 and 100 μM) was essentially inactive (Figure 2a). In contrast, rolipram potently suppressed cyclic AMP hydrolysis in the soluble fraction of T-cell lysates (EC_{50} = 176.2 ± 41.9 and 330.2 ± 78.1 nM for $CD4^+$ and $CD8^+$ T-cells respectively), while SK&F 95654 was only weakly active at the concentrations (10 and 100 μM) examined (Figure 2b). A consistent finding in these experiments was that rolipram did not abolish cyclic AMP hydrolysis. Indeed, in both $CD4^+$ and $CD8^+$ T-cell subsets the maximum inhibition that was achieved amounted to ~65% (Figure 2b) suggesting that more than one cyclic AMP PDE was present in the soluble fraction.

Table 1 Primers and conditions used in RT-PCR experiments

*Gene**	*Deoxyoligonucleotide sequences*	*Fragment sizes (base pairs)*	*Denaturing temperature*	*Annealing temperature*	*Extension temperature*
HSPDE4A	forward 5′-AAG AGG AGG AAG AAA TAT CAA TGG-3′ reverse 5′-TTA CAG CAA CCA CGA ATT CCT CC-3′	272	94°C for 30 s	67°C for 30 s	72°C for 60 s
HSPDE4B	forward 5′-AGG GCA GCC TGA GGT ATT AAA-3′ reverse 5′-CAC TCC TGG CTT ACA GTT GTA-3′	363	94°C for 30 s	62°C for 30 s	72°C for 60 s
HSPDE4C	forward 5′-GGA GTT GCC TGA CAC TGA ACT-3′ reverse 5′-AGA AAG ACA CCA GGG CAT CGT-3′	335	94°C for 30 s	71°C for 30 s	72°C for 60 s
HSPDE4D	forward 5′-GCA AGA TCG AGC ACC TAG CAA-3′ reverse 5′-CGG TTA CCA GAC AAC TCT GTC-3′	515	94°C for 30 s	62°C for 30 s	72°C for 60 s
HSPDE7	forward 5′-GGA CGT GGG AAT TAA GCA AGC-3′ reverse 5′-TCC TCA CTG CTC GAC TGT TCT-3′	285	94°C for 30 s	59°C for 30 s	72°C for 60 s
GAPDH	forward 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ reverse 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′	598	94°C for 30 s	58°C for 30 s	72°C for 60 s

*PDE nomenclature of Beavo *et al.* (1994)

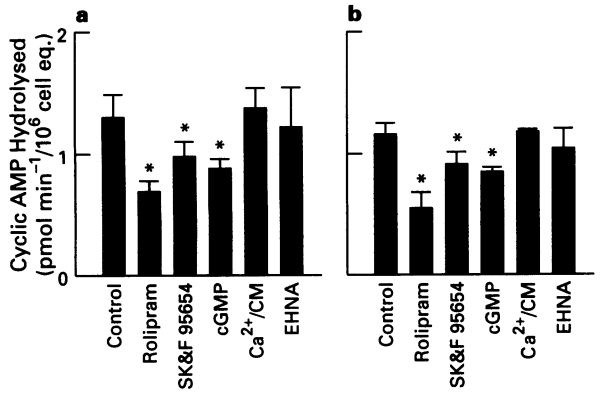

Figure 1 Effect of allosteric modulators and isoenzyme-selective PDE inhibitors upon cyclic AMP hydrolysis in lysates of human peripheral blood T-lymphocytes. (a) $CD4^+$ and (b) $CD8^+$ T-cells were suspended in hypotonic buffer and PDE activity in aliquots of the resulting lysate was measured at a substrate concentration of 1 μM cyclic AMP in the absence and presence of rolipram (10 μM), SK&F 95654 (10 μM), cyclic GMP (10 μM), calmodulin (50 units plus 2 mM Ca^{2+}) and EHNA (1 μM). Each column represents the mean of four independent determinations performed with different cell preparations; vertical lines show s.e.mean. See Methods for further details. *$P<0.05$ – significant inhibition of cyclic AMP PDE activity relative to control rate.

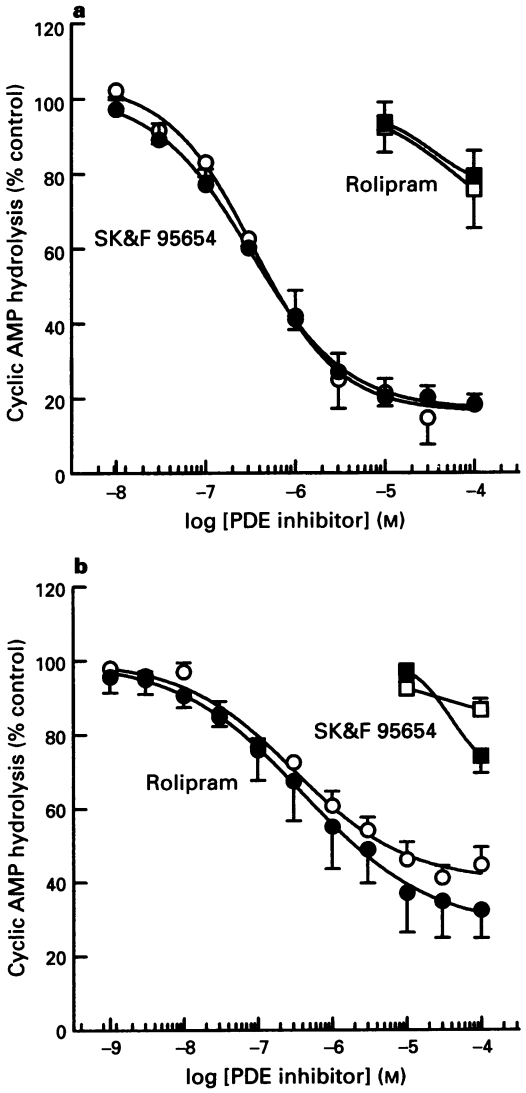

Figure 2 Effect of rolipram and SK&F 95654 on cyclic AMP hydrolysis in the particulate (a) and soluble (b) fraction of human peripheral blood T-lymphocytes. $CD4^+$ (■ ●) and $CD8^+$ (□ ○) T-cells were suspended for 30 min in hypotonic buffer and centrifuged at 145,000 *g* for 30 min at 4°C. The ability of rolipram and SK&F 95654 to inhibit cyclic AMP hydrolysis in the resulting particulate and soluble fraction was then assessed at a substrate concentration of 1 μM. Each data point represents the mean of six independent determinations performed with cell preparations from different donors; vertical lines show s.e.mean. See Methods for further details.

In agreement with data obtained in human eosinophils (Dent *et al.*, 1994) and monocytes (Seldon *et al.*, 1995), the concentration-response curves which described the inhibition of PDE4 by rolipram were shallow with mean pseudo Hill coefficients ($CD4^+$: -0.513 ± 0.098; $CD8^+$: -0.575 ± 0.071) significantly ($P<0.05$) less than unity (Figure 2b). SK&F 95654, in contrast, inhibited membrane-bound PDE3 in $CD4^+$ and $CD8^+$ T-cells with slopes of -0.839 ± 0.111 and -1.007 ± 0.093, respectively (Figure 2a).

Significantly, the residual rolipram-insensitive component in the soluble fraction was not ascribable to PDE2, PDE3 or PDE5 since EHNA (1 μM), cyclic GMP (10 μM), SK&F 95654 (10 μM) and zaprinast (10 μM) were without effect. Qualitatively identical results were obtained in the particulate fraction of T-cells where PDE3 predominated. In these studies, however, the SK&F 95654-insensitive activity was relatively modest (~15%) but, nevertheless, was similarly resistant to representative inhibitors of the other PDE isoenzyme families.

Kinetic analysis of cyclic AMP hydrolysis in the soluble and particulate fraction of $CD4^+$ and $CD8^+$ T-lymphocytes

The hydrolysis of cyclic AMP in the particulate fraction of T-lymphocytes conformed to Michealis-Menten kinetic behaviour as evinced by the linear plots of Hofstee transformed data (Figure 3 and inset). There was no significant difference ($P>0.05$) between $CD4^+$ and $CD8^+$ T-lymphocytes with respect to either the K_m ($CD4^+$: 0.26 ± 0.06 μM; $CD8^+$: 0.34 ± 0.05 μM) or V_{max} ($CD4^+$: 0.74 ± 0.21; $CD8^+$: 0.81 ± 0.34 pmol min^{-1}/10^6 cell eq.) of cyclic AMP hydrolysis. In contrast, cyclic AMP PDE activity in the soluble fraction of T-lymphocytes did not conform to Michealis-Menten kinetic behaviour and Hofstee analyses yielded curvilinear plots suggesting the presence of either two distinct enzyme activities or a single enzyme with two catalytic domains (Figure 4). Resolution of these data into two components, assuming conformation of both activities to Michealis-Menten kinetic behaviour, revealed a high affinity (K_m: $CD4^+=0.09\pm0.02$ μM; $CD8^+=0.11\pm0.03$ μM, $n=3$; $P>0.05$), low capacity (V_{max}: $CD4^+$: 0.51 ± 0.14; $CD8^+=0.58\pm0.19$ pmol min^{-1}/10^6 cell eq., $n=3$; $P>0.05$) activity and a low affinity (K_m: $CD4^+=3.6\pm0.9$ μM; $CD8^+=2.3\pm0.7$ μM, $n=3$; $P>0.05$), high capacity (V_{max}: $CD4^+=3.27\pm0.34$; $CD8^+=4.01\pm0.55$ pmol min^{-1}/10^6 cell eq., $n=3$; $P>0.05$) activity (Figure 4, inset).

Expression of HSPDE4 and HSPDE7 mRNAs in $CD4^+$ and $CD8^+$ T-lymphocytes

The presence of mRNAs for the PDE4 subtypes expressed in human T-lymphocytes was evaluated by RT-PCR with primers designed to recognise unique sequences in the four human genes (Bolger *et al.*, 1993). In view of the large amount of rolipram- and SK&F 95654-resistant cyclic AMP hydrolytic activity in both T-cell subsets, an identical approach was employed to assess whether the recently discovered HSPDE7 gene (Michaeli *et al.*, 1993) is also transcribed by these cells. Figure 5 shows an ethidium bromide-stained agarose gel of a representative experiment. In four independent determinations with cells from different subjects, staining of gels for

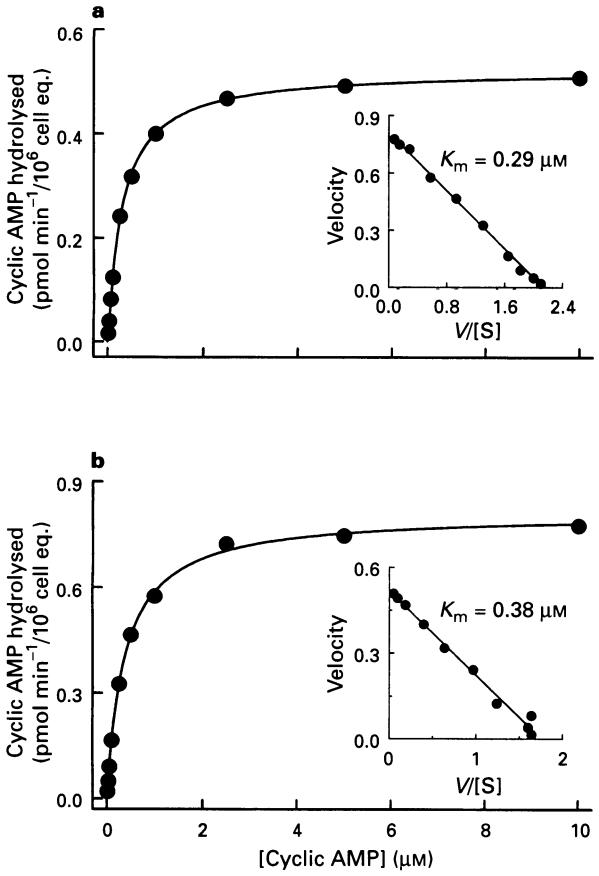

Figure 3 Kinetics of cyclic AMP hydrolysis in the particulate fraction of human peripheral blood T-lymphocytes. $CD4^+$ (a) and $CD8^+$ (b) T-cells were suspended for 30 min in hypotonic buffer and centrifuged at 145,000*g* for 30 min at 4°C. PDE activity was measured in the particulate fraction at substrate concentrations ranging from 0.1 to 10 μM cyclic AMP. The data were subsequently transformed according to Hoftsee (insets) from which estimates of K_m and V_{max} were derived. The results are representative of three independent experiments conducted with cell preparations from different donors. See Methods for further details.

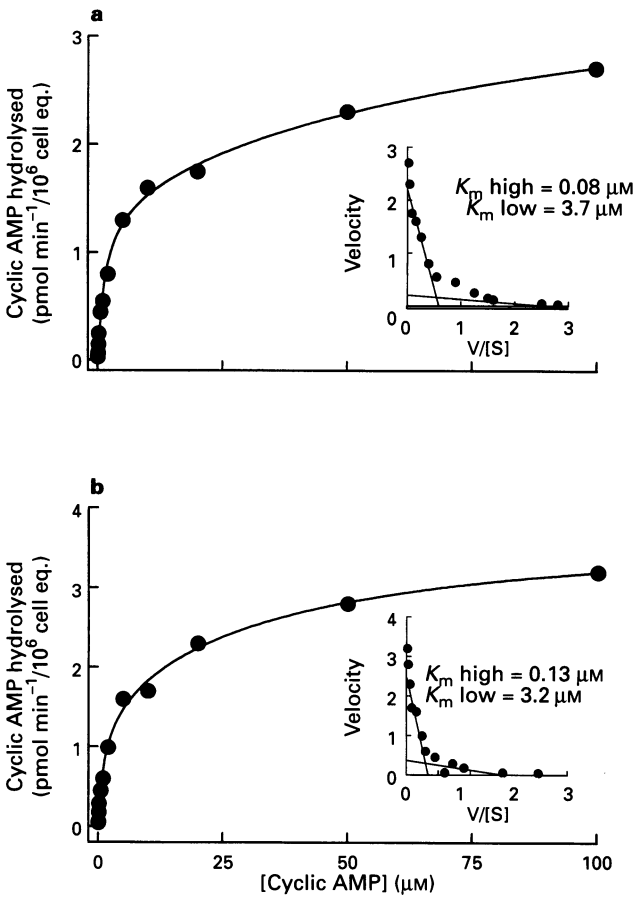

Figure 4 Kinetics of cyclic AMP hydrolysis in the soluble fraction of human peripheral blood T-lymphocytes. $CD4^+$ (a) and $CD8^+$ (b) T-cells were suspended for 30 min in hypotonic buffer and centrifuged at 145,000*g* for 30 min at 4°C. PDE activity was measured in the soluble fraction at substrate concentrations ranging from 0.1 to 100 μM cyclic AMP. Data were subsequently transformed according to Hoftsee (insets) and resolved into two components according to Spears *et al.* (1971) from which estimates of K_m and V_{max} were then derived for the high and low affinity activities. The results are representative of three independent experiments conducted with cell preparations from different donors. See Methods for further details.

RT-PCR products of human $CD4^+$ and $CD8^+$ T-lymphocyte total RNA revealed amplified cDNA fragments corresponding to the predicted sizes of HSPDE4A (272 bp), HSPDE4B (363 bp), HSPDE4D (515 bp) and HSPDE7 (285 bp) which was subsequently confirmed by cloning of the PCR products into pGEM5z (Promega, Southampton) followed by double stranded sequencing with Sequenase II (Amersham International, Buckinghamshire). HSPDE4C mRNA was not detected in T-cells from any subject after 35 cycles of amplification.

A consistent finding in these experiments was the presence of a product from genomic DNA of identical size to that derived from the cDNA for HSPDE4A and HSPDE4C (Figure 5), suggesting a lack of introns between the primers used for the PCR. It was therefore necessary to eliminate the possibility that this may have arisen from genomic contamination.Thus an aliquot of the same RNA (1 μg) was run in parallel under conditions used for the RT-PCR reaction but in the absence of reverse transcriptase. Under these conditions, PCR of the samples, performed in parallel with the regular RT products, did not show a detectable band on agarose gels for any PCR primer pair indicating that the bands seen for amplified cDNA were not due to genomic contamination (data not shown). This conclusion was supported by the finding that no specific bands of amplified genomic DNA were detected for HSPDE4B, HSPDE4D, HSPDE7 and GAPDH.

Effect of rolipram and SK&F on the cyclic AMP content of $CD4^+$ and $CD8^+$ T-lymphocytes

The basal cyclic AMP content in $CD4^+$ and $CD8^+$ T-lymphocytes was 0.73 ± 0.22 ($n = 10$) and 0.75 ± 0.09 pmol/10^6 cells ($n = 10$), respectively ($P > 0.05$). Intriguingly, although appreciable amounts of PDE3 and PDE4 were expressed by these cells (see above), only rolipram evoked a detectable increase in the cyclic AMP content (Figure 6). This effect was concentration-dependent and, at 100 μM rolipram, resulted in a three to four fold increase in cyclic AMP mass to 2.97 ± 1.14 and 2.26 ± 0.32 pmol/10^6 cells for $CD4^+$ and $CD8^+$ T-cells, respectively (Figure 6). No significant difference in basal or rolipram-stimulated cyclic AMP accumulation was noted between $CD4^+$ and $CD8^+$ T-lymphocytes.

Effect of rolipram and SK&F 95654 on the proliferation of $CD4^+$ and $CD8^+$ T-lymphocytes

T-Lymphocyte proliferation was assessed by monitoring the incorporation of [^{3}H]-thymidine into cellular DNA in response to submaximal concentrations of three established mitogens: anti-CD3 (500 ng ml^{-1}), PHA (5 μg ml^{-1}) and a combination of PMA and ionomycin (1 μM each). At the concentrations employed, each mitogen was essentially equi-effective and sti-

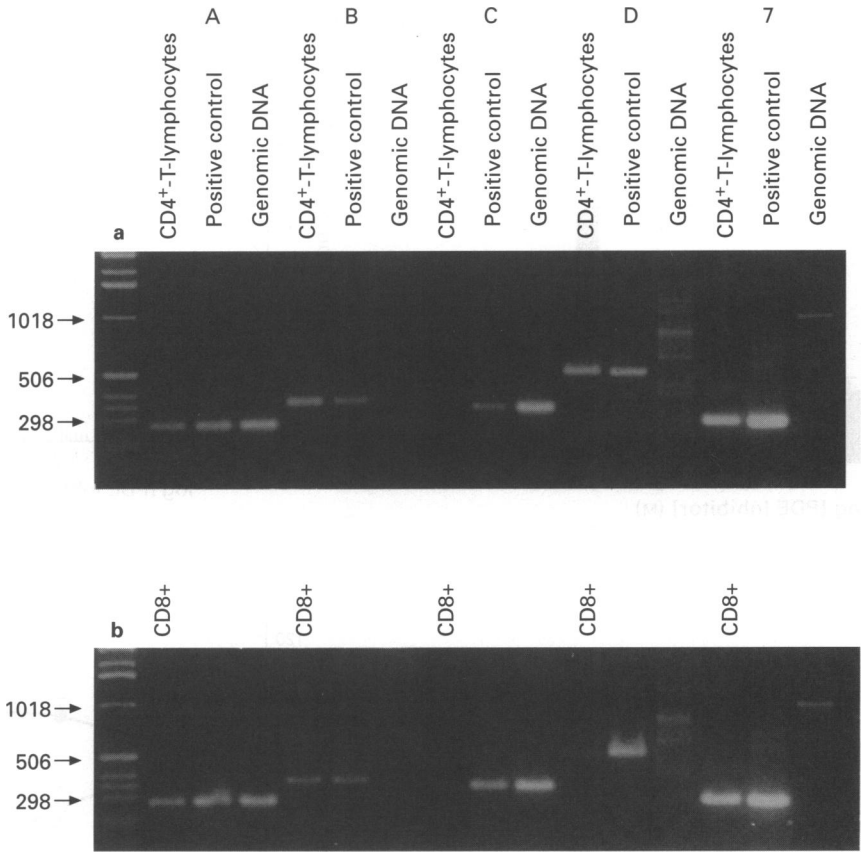

Figure 5 Representative ethidium bromide-stained agarose gels of PDE4 and PDE7 message amplification fragments in human peripheral blood $CD4^+$ (a) and $CD8^+$ (b) T-lymphocytes. Total RNA was extracted from approximately 5×10^6 T-cells and 1 μg was reverse transcribed to generate cDNAs for PDE7 and the PDE4 isogenes with specific primers (Table 1). PCR was then performed (35 cycles) with reverse transcribed cDNA, the products subjected to electrophoresis on 2% agarose gels and DNA subsequently visualised by ethidium bromide staining. Positive controls (cDNA from total human lung RNA or 1 ng of the appropriate cDNA cloned into the vector pGEM5z) and 100 ng human genomic DNA were routinely run in parallel. RT-PCR product sizes for HSPDE4A, HSPDE4B, HSPDE4C, HSPDE4D and HSPDE7 were 272 bp, 363 bp, 335 bp, 515 bp and 285 bp respectively. The left hand lane in each gel shows molecular weight markers (0.5 μg 1 kb ladder, Gibco). The gels are representative of four experiments conducted with cells obtained from different donors. See Methods for further details.

mulated the uptake of 40000 to 60000 d.p.m. of [^{3}H]-thymidine into both T-cell subsets at 72 h over a baseline level of 100 to 300 d.p.m.

Culture of T-cells with rolipram inhibited PHA-induced incorporation of [^{3}H]-thymidine (Figure 7). This effect was concentration-dependent with IC_{50} values of 10.6 ± 2.6 μM and 5.2 ± 1.7 μM ($P > 0.05$; $n = 14$) for $CD4^+$ and $CD8^+$ T-cells respectively., Qualitatively identical data were obtained when anti-CD3 was used as mitogen (IC_{50} $CD4^+$: 4.9 ± 2.7 μM; $CD8^+$: 2.31 ± 0.63 μM, $P > 0.05$; $n = 5$; Figure 8).

In contrast, SK&F 95654 failed to suppress PHA- and anti-CD3-induced [^{3}H]-thymidine uptake at any concentration examined (Figures 7 and 8) although, as illustrated in Figure 9, it significantly ($P < 0.01$) potentiated the inhibitory effect of rolipram on PHA-induced T-cell proliferation such that the IC_{50} of rolipram was reduced approximately 50 to 100 fold from 14.2 ± 4.5 to 0.12 ± 0.03 μM ($n = 7$) and from 10.2 ± 2.1 to 0.23 ± 0.05 μM ($n = 7$) for $CD4^+$ and $CD8^+$ T-cells, respectively.

When T-cell proliferation was induced by the co-addition of PMA and ionomycin, neither SK&F 95654 nor rolipram suppressed the incorporation of [^{3}H]-thymidine at any concentration examined (Figure 10). The ability of rolipram to prevent proliferation was thus dependent upon the nature of the activating stimulus.

In all experiments cell viability, measured by Trypan blue exclusion, was maintained at >95% throughout the incubation period and was not affected by any of the mitogens examined or either rolipram of SK&F 95654 (data not shown).

Effect of rolipram and SK&F 95654 on the generation of IL-2 and IFNγ by $CD4^+$ and $CD8^+$ T-lymphocytes

At rest, $CD4^+$ and $CD8^+$ T-lymphocytes released little if any IL-2 or IFNγ (10 to 20 pg ml^{-1}). However, following the addition of PHA (5 μg ml^{-1}) the generation of these cytokines increased significantly (IL-2: range 54.4 to 100.2 pg ml^{-1}; INFγ: range 134.3 to 1294 pg ml^{-1}) measured at 12 h. Figure 11 illustrates that culture of both T-cell subsets with rolipram partially inhibited these responses by 40 to 60% at the highest concentration examined with the exception of the release of IFNγ from $CD4^+$ T-cells which was, inexplicably, more sensitive to rolipram (Figure 11a).

The inhibition of PHA-induced IL-2 release from $CD4^+$ T-lymphocytes by rolipram (Figure 12) was concentration-dependent with an IC_{50} (178.2 ± 63.3 nM, $n = 7$) in good agreement with its potency at inhibiting PDE4 in the soluble fraction of $CD4^+$ T-cell lysates but 60 times lower than for the suppression of PHA-induced proliferation (Figure 7a). Interestingly, ~20% of the PHA-induced response was resistant to rolipram (Figure 12). Culture of T-cells with SK&F 95654 (10 μM) did not affect the release of either IL-2 or IFNγ under identical experimental conditions which was consistent with the inability of the PDE3 inhibitor to suppress PHA-induced [^{3}H]-thymidine uptake (Figures 11 and 12) and to elevate the cyclic AMP content (Figure 6).

Culture of $CD4^+$ T-lymphocytes with SK&F 95654 (10 μM) which, as stated above, did not affect the release of

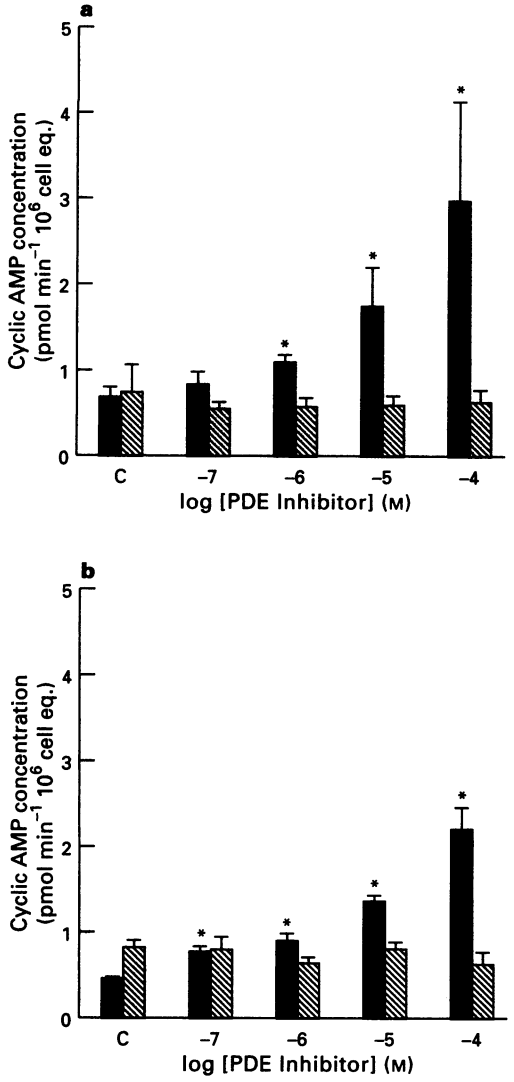

Figure 6 Effect of rolipram and SK&F 95654 on the cyclic AMP content of human peripheral blood T-lymphocytes. $CD4^+$ (a) and $CD8^+$ (b) T-cells were incubated for 10 min at 37°C in buffer A and then exposed to either rolipram (100 nM to 100 μM, solid columns), SK&F 95654 (100 nM to 100 μM, hatched columns) or vehicle for a further 10 min. The reaction was quenched by the addition of an equal volume of TCA (1 M) and cyclic AMP subsequently extracted, neutralised and measured by RIA as detailed in Methods. Each column represents the mean of 10 independent experiments conducted with cell preparations from different donors; vertical lines show s.e.mean. *$P<0.05$ – significant increase in cyclic AMP mass relative to corresponding basal level.

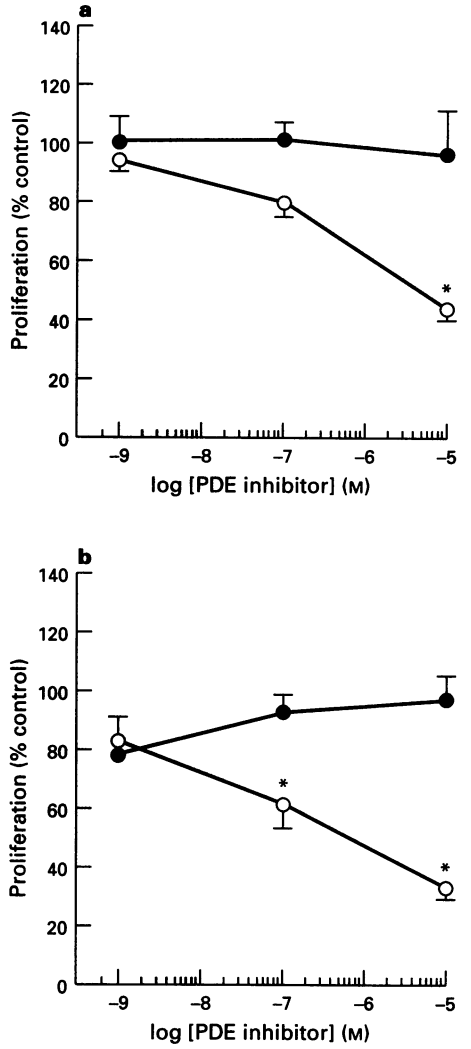

Figure 7 Effect of rolipram (○) and SK&F 95654 (●) on PHA-induced [^{3}H]-thymidine incorporation into human peripheral blood T-lymphocytes. Highly purified $CD4^+$ (a) and $CD8^+$ (b) T-cells were cultured with rolipram (1 nM to 10 μM) or SK&F 95654 (1 nM to 10 μM) in supplemented RPMI 1640 containing PHA (5 μg ml^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere for 64 h at which time 0.5 μCi [^{3}H]-thymidine was added to each well. After a further 8 h incubation the amount of radioactivity taken up by the T-cells was measured by liquid scintillation counting. Basal and PHA-induced [^{3}H]-thymidine incorporation were 301 ± 27 and 41719 ± 9538, and 238 ± 52 and 63056 ± 10938 d.p.m. for $CD4^+$ and $CD8^+$ T-cells respectively. Data represent the mean of 14 independent experiments conducted with different cell preparations; vertical lines show s.e.mean. See Methods for further details. *$P<0.05$ – significant inhibition of PHA-induced [^{3}H]-thymidine incorporation.

either cytokine studied, significantly potentiated the inhibitory effect of rolipram (Figure 12). Thus, there was a leftwards-shift of the concentration-response curve which described the inhibition of IL-2 generation and an associated 20 fold increase in the apparent potency of rolipram ($IC_{50} = 6.1 \pm 3.3$ nM, $n = 7$).

Since rolipram failed to suppress the proliferation of T-cells when PMA and ionomycin were used as a mitogenic stimulus (Figure 10), parallel experiments were performed to assess if this could be due to an inability of PDE4 inhibitors to prevent IL-2 generation. However, as shown in Figure 11, rolipram (10 μM) markedly reduced the elaboration of IL-2 from $CD4^+$ and $CD8^+$ T-cells by an amount similar to when PHA was used as stimulus (see above). Rolipram was similarly effective at attenuating the generation of IFNγ (Figure 11).

Discussion

By use of a combination of biochemical, pharmacological and molecular techniques, evidence was obtained that $CD4^+$ and $CD8^+$ T-lymphocytes purified from the peripheral blood of normal, non-atopic subjects express three kinetically distinct cyclic AMP PDE activities that have the characteristics of PDE3, PDE4 and PDE7 isoenzymes. In none of the studies performed was there evidence for a difference between the T-cell subsets. Subcellular distribution studies demonstrated that the particulate (145,000 × g) fraction contained essentially a kinetically homogeneous PDE which exhibited a number of characteristics consistent with a PDE3 isoenzyme. Thus, the hydrolysis of cyclic AMP conformed to Michealis-Menten

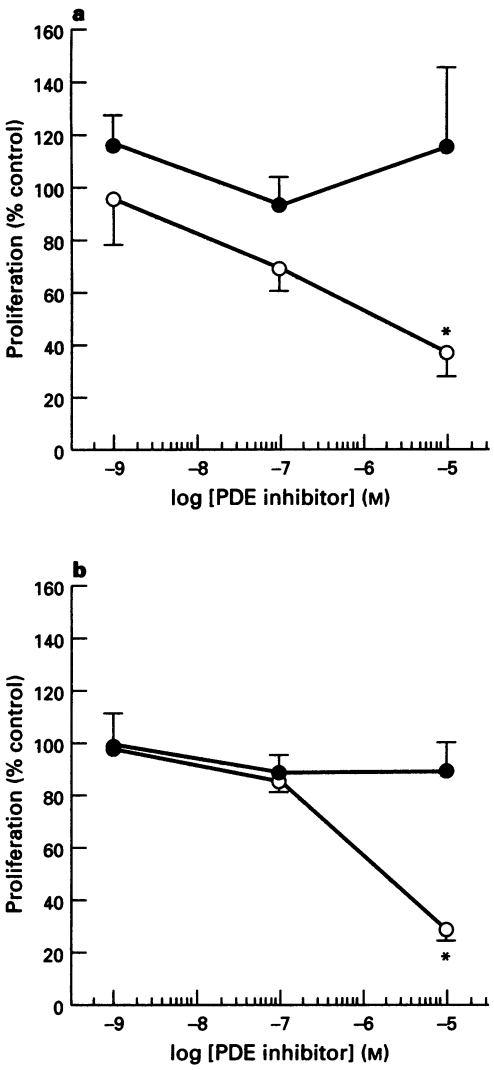

Figure 8 Effect of rolipram (●) and SK&F 95654 (○) on anti-CD3-induced [^{3}H]-thymidine incorporation into human peripheral blood T-lymphocytes. Highly purified CD4$^+$ (a) and CD8$^+$ (b) T-cells were cultured with rolipram (1 nM to 10 μM) or SK&F 95654 (1 nM to 10 μM) in supplemented RPMI 1640 containing anti-CD3 (500 ng ml^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere for 64 h at which time 0.5 μCi [^{3}H]-thymidine was added to each well. After a further 8 h incubation the amount of radioactivity taken up by the T-cells measured by liquid scintillation counting. Basal and PHA-induced [^{3}H]-thymidine incorporation were 313 ± 25 and 41381 ± 13628, and 241 ± 21 and 33340 ± 9927 d.p.m. for CD4$^+$ and CD8$^+$ T-cells, respectively. Data represent the mean of five independent experiments conducted with differenct cell preparations; vertical lines show s.e.mean. See Methods for further details. *P<0.05–significant inhibition of anti-CD3-induced [^{3}H]-thymidine incorporation.

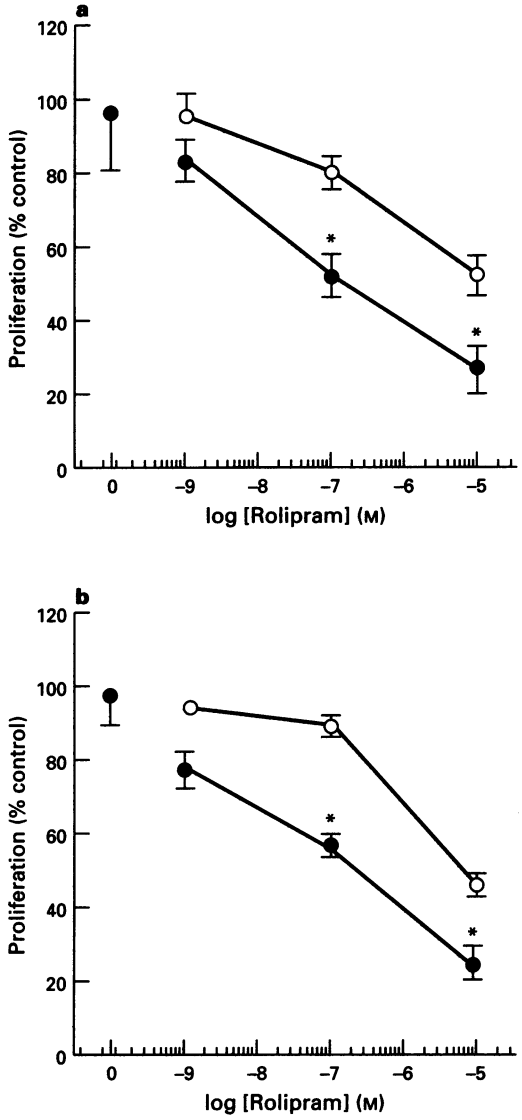

Figure 9 Effect of SK&F 95654 on the ability of rolipram to suppress PHA-induced [^{3}H]-thymidine incorporation into human peripheral blood T-lymphocytes. Highly purified CD4$^+$ (a) and CD8$^+$ (b) T-cells were cultured with rolipram (1 nM to 10 μM) in the absence (○) and presence (●) of SK&F 95654 (10 μM) in supplemented RPMI 1640 containing PHA (5 μg ml^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere for 64 h at which time 0.5 μCi [^{3}H]-thymidine was added to each well. After a further 8 h incubation the amount of radioactivity taken up by the T-cells was measured by liquid scintillation counting. Data represent the mean of seven independent experiments conducted with different cell preparations; vertical lines show s.e.mean. See Methods for further details. *P<0.05–significant potentiation of the inhibitory effect induced by rolipram.

kinetic behaviour with a K_m of ~200 nM. In addition, SK&F 95654, a selective inhibitor of the PDE3 isoenzyme family (Murray *et al.*, 1992), suppressed PDE activity with an IC_{50} (~0.5 to 1 μM) in excellent agreement with its potency against other PDE3 preparations (Murray *et al.*, 1992). In contrast, the major PDE in the soluble fraction of T-cell lysates, which accounted for approximately 65% of the total activity, was attributed to a PDE4; this enzyme had a K_m for cyclic AMP of ~4 μM and was effectively suppressed by rolipram with a potency (IC_{50} ~100–300 nM) similar to that obtained for the inhibition of PDE4 in human eosinophils (Dent *et al.*, 1994) and for human monocyte PDE4A expressed in yeast (Torphy *et al.*, 1992). Collectively, these data confirm the differential distribution of PDE3 and PDE4 between the soluble and particulate fraction in an enriched population of CD3$^+$ T-lymphocytes (Robiscek *et al.*, 1991) and are entirely consistent with the more recent finding of the sub-cellular localisation of PDE isoenzymes in highly purified CD4$^+$ and CD8$^+$ human T-cell subsets (Tenor *et al.*, 1995).

The PDE4 family comprises a markedly heterogeneous group of proteins. Currently, greater than 16 enzymes can theoretically be derived from transcription of four distinct genes (see Introduction). It is possible that inhibition of one or more PDE4 isoenzymes underlies some of the adverse effects (e.g. nausea, vomiting) that are commonly associated with non-selective PDE4 inhibitors, and emphasizes the need to identify the PDE4 genes that are transcribed by the target cells of interest and to synthesize and evaluate the functional effects

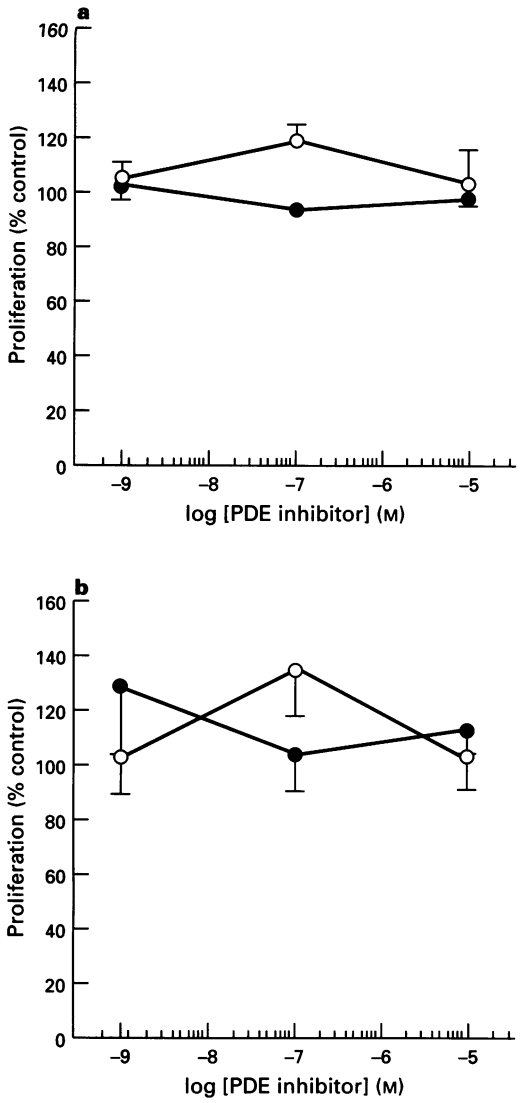

Figure 10 Effect of rolipram (○) and SK&F 95654 (●) on [^{3}H]-thymidine incorporation into human peripheral blood T-lymphocytes in response to PMA and ionomycin. Highly purified CD4$^+$ (a) and CD8$^+$ (b) T-cells were cultured with rolipram (1 nM to 10 μM) or SK&F 95654 (1 nM to 10 μM) in supplemented RPMI 1640 containing PMA (1 μM) and ionomycin (1 μM). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere for 64 h at which time 0.5 μCi [^{3}H]-thymidine was added to each well. After a further 8 h incubation the amount of radioactivity taken up by the T-cells was measured by liquid scintillation counting. Basal and PHA-induced [^{3}H]-thymidine incorporation were 119±18 and 46707±7244, and 235±22 and 50014±7831 d.p.m. for CD4$^+$ and CD8$^+$ T-cells, respectively. Data represent the mean of seven independent experiments conducted with different cell preparations; vertical lines show s.e.mean. See Methods for further details.

of sub-family-selective inhibitors. We have begun to study the complement of PDE4 isogenes in pro-inflammatory cells implicated in the pathogenesis of asthma and have unambiguously identified, by use of RT-PCR of total RNA extracted from human CD4$^+$ and CD8$^+$ T-lymphocytes, mRNA for PDE4A, PDE4B and PDE4D. This distribution is identical to the PDE4 mRNAs found in human eosinophils and monocytes but, significantly, is at variance with the complement of PDE4 isoenzymes expressed by the Jurkat human T-cell line, which apparently express mRNA for the PDE4A isoform only (Engels *et al.*, 1994). This is, perhaps, a surprising finding and highlights the potential limitations of using cell lines as models of peripheral blood leukocytes when PDE4 is the protein of interest.

A consistent finding of these studies was the presence of a large amount (~35%) of a rolipram- and SK&F 95654-insensitive PDE activity in the soluble fraction of CD4$^+$ and CD8$^+$ T-lymphocytes that was not affected by selective inhibitors of the other PDE families. Moreover, the hydrolysis of cyclic AMP in the soluble fraction did not conform to Michealis-Menten kinetic behaviour and analysis of the data by the method of Spears *et al.* (1971) resolved two distinct PDE activities. The identification of an atypical PDE in peripheral blood T-lymphocytes is reminiscent of an earlier study in which a similar activity, denoted JK-21, was partially purified and characterized from Jurkat, MOLT-4, HUT 78 and HBP-ALL human T-cell lines (Ichimura & Kase, 1993). Indeed, the peripheral blood CD4$^+$ and CD8$^+$ T-cell enzymes have similar kinetic constants and substrate specificity to JK-21. In addition, this novel activity shows remarkable similarity to PDE7, a rolipram-insensitive, high affinity cyclic AMP PDE that was recently identified from the screening of a human glioblastoma cDNA library (Michaeli *et al.*, 1993). Although it is not possible unequivocally to attribute the rolipram-resistant cyclic AMP PDE activity to PDE7 (owing to the lack of selective inhibitors), compelling evidence to support this contention was provided by the unambiguous identification of PDE7 mRNA in both T-cell subsets.

Culture of CD4$^+$ and CD8$^+$ T-cells with rolipram resulted in inhibition of PHA- and anti-CD3-induced proliferation as assessed by the cellular uptake of [^{3}H]-thymidine when compared to vehicle-treated cells. This finding is consistent with the ability of the PDE4 inhibitor, Ro 20-1724, to prevent PHA-induced mitogenesis of a mixed population of T-lymphocytes (Averill *et al.*, 1988; Robicsek *et al.*, 1991) and demonstrates a functional role for a PDE4 isoenzyme in regulating T-cell growth and division. Surprisingly, these data are at variance with a recent study from Essayan *et al.* (1994) in which rolipram failed to inhibit PHA-induced [^{3}H]-thymidine uptake by human PBMCs harvested from atopic subjects sensitized to ragweed. The explanation for this disparity is elusive especially since antigen-driven proliferation was significantly suppressed by rolipram in that study. Further experiments performed by Essayan *et al.* (1994) indicate that these results do not reflect a difference between normal and atopic subjects which leaves the possibility (aside from differences in methodology between the two studies) that the monocyte component (~20%) of the mononuclear cell preparation may, in some unknown way, render ragweed-sensitive T-lymphocytes refractory to rolipram.

Although T-lymphocytes expressed a high level of a PDE3 isoenzyme(s), SK&F 95654 failed to suppress PHA- and anti-CD3-induced [^{3}H]-thymidine uptake under identical experimental conditons. The reason for this inactivity is unclear, but a number of explanations could account for these results. One possibility may relate to the fact that PDE3 was localised exclusively to the membrane fraction which may render it unable to affect those proteins which govern proliferation. Although evidence for functional compartmentalisation of cyclic nucleotide action is available in other cell types, this explanation is inconsistent with the ability of SK&F 95654 to potentiate the anti-proliferative effect of rolipram. In fact, these data argue against this hypothesis and indicate that inhibition of PDE3 can, indeed, prevent mitogenesis of T-cells provided cyclic AMP is simultaneously elevated by another stimulus. This may indicate that PDE4 needs to be inhibited before SK&F 95654 is able to gain access to and/or suppress cyclic AMP hydrolysis by PDE3. It is noteworthy, that a synergistic interaction between inhibitors of PDE3 and PDE4 has been documented in other cells which express representatives of these PDE families including rat thymocytes (Marcoz *et al.*, 1993), human platelets (Maurice & Haslam, 1990) and an enriched population of CD3$^+$ T-lymphocytes (Robicsek *et al.*, 1991), and appears to represent a generalised rather than a tissue-specific phenomenon.

Another hypothesis that can be formulated for the failure of SK&F 95654 to inhibit proliferation is based upon the fact that

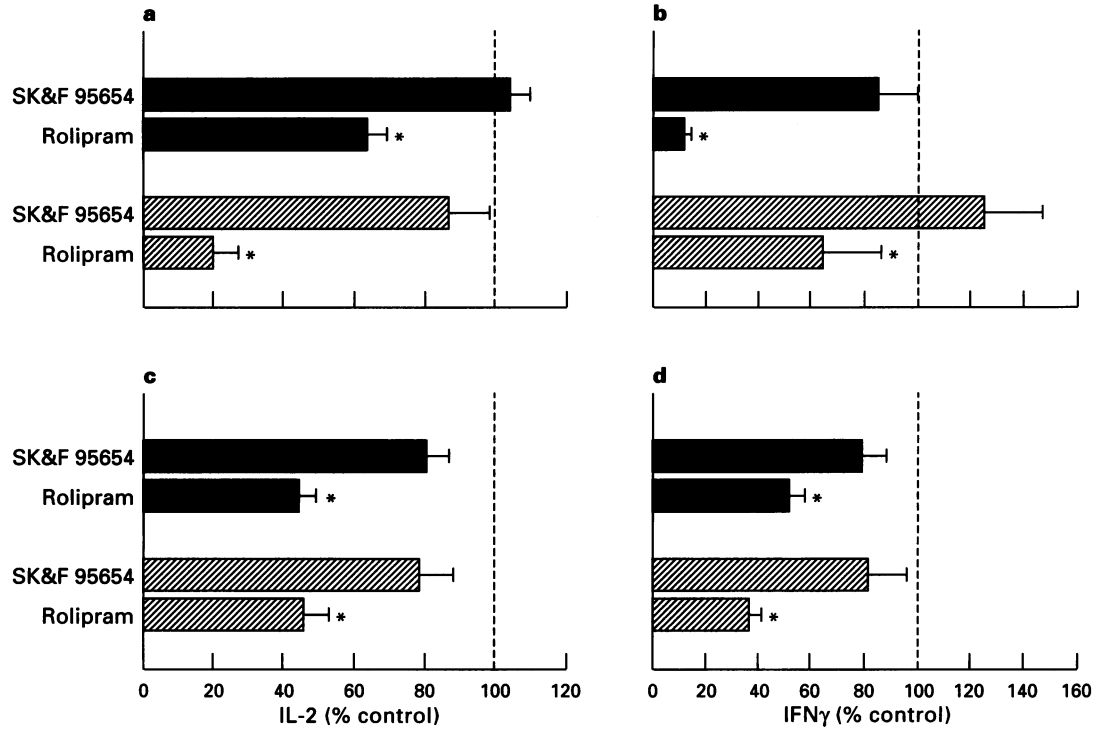

Figure 11 Effect of rolipram and SK&F 95654 on mitogen-evoked generation of IL-2 and IFNγ by human peripheral blood T-lymphocytes. Highly purified $CD4^+$ (a and b) and $CD8^+$ (c and d) T-cells were cultured with rolipram (10 μM) or SK&F 95654 (10 μM) in supplemented RPMI 1640 and exposed to PHA (5 μg ml^{-1}; solid columns) or a combination of PMA (1 μM) and ionomycin (1 μM; hatched columns). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of IL-2 and IFNγ released into the culture medium was measured at 16 h by a specific ELISA. Data represent the mean ± s.e.mean of seven independent experiments conducted with different cell preparations. See Methods for further details. *$P<0.05$ – significant inhibition of mitogen-induced cytokine generation.

the V_{max} of PDE3-catalysed cyclic AMP hydrolysis was five-times lower than the same reaction catalysed by PDE4. It is possible, therefore, that selective inhibition of PDE3 would not result in an increment in cyclic AMP of sufficient magnitude to exert an anti-mitogenic effect. Indeed, this postulate is supported by the finding that SK&F 95654 failed detectably to increase the cyclic AMP content in either T-cell subset at a concentration 100-times higher than the IC_{50} for inhibition of partially purified PDE3. In addition, the affinity of cyclic AMP for PDE4 ($K_m \sim 3$ μM) is at least 10-times lower than for PDE3 ($K_m \sim 0.2$ μM). Thus, as the cyclic AMP concentration increases PDE4 becomes the predominant hydrolysing activity and, therefore, functionally the most important.

Compelling evidence is available that T-lymphocyte proliferation is triggered by the activation of an autocrine and/or paracrine system that requires the prior generation and release of IL-2 together with the expression of the IL-2R with which it subsequently interacts. In its simplest form, activation of the T-cell receptor (TCR) promotes a transition of the cell from a state of rest (i.e. G_0) to the G_1 phase of the cell cycle with associated IL-2 generation and interleukin-2 receptor (IL-2R) expression. Progression of T-cells into the S phase, a phenotype characterized by DNA synthesis, occurs several hours later at a time when sufficient IL-2 molecules have interacted with their cognate receptors. It is clear from this description that cyclic AMP-elevating agents could suppress the proliferative response by interfering with TCR and/or IL-2R signalling. Previous studies have provided evidence that cyclic AMP inhibits T-cell proliferation primarily by inhibiting IL-2R-mediated G_1 progression into the S phase of the cell cycle (Johnson *et al.*, 1988; Lingk *et al.*, 1990). However, our results suggest that TCR-mediated events also represent a primary target since rolipram suppressed the generation of IL-2 (and IFNγ) by $CD4^+$ and $CD8^+$ T-lymphocytes in response to PHA and anti-CD3. Moreover, in complete agreement with the proliferation data described above, SK&F 95654, while inactive alone, significantly potentiated this effect of rolipram indicating a role for the PDE3 and PDE4 isoenzyme families in regulating IL-2 gene expression. These observations are, therefore, consistent with an inhibitory action of cyclic AMP on mitogen-induced IL-2 gene induction and, theoretically, may reflect the ability of cyclic AMP to block a number of events implicated in mitogenesis. These include phospholipase Cγ1 (PLCγ1)-induced phosphatidylinositol (4,5)bisphosphate ($PtdIns(4,5)P_2$) hydrolysis (Tamir & Isakov, 1994) and inositol phosphate accumulation (Takayama *et al.*, 1988; Granja *et al.*, 1991; Park *et al.*, 1992), Ca^{2+} mobilisation (Van Tits *et al.*, 1991), the phosphorylation of the TCR (Patel *et al.*, 1987; Klausner *et al.*, 1987), interleukin-2 receptor (IL-2R) expression (Krause & Deutsch, 1991) and the transcription of the interleukin-2 (IL-2) gene through *cis*-regulatory elements that may include the NF-AT, NFκB and TGGGC domains on the IL-2 promoter (Novak & Rothenberg, 1990; Chen & Rothenberg, 1994; Tsurata *et al.*, 1995).

In contrast to PHA- and anti-CD3-driven mitogenesis, the increase in [^{3}H]-thymidine incorporation evoked by a combination of PMA and ionomycin in $CD4^+$ and $CD8^+$ T-lymphocytes was not inhibited by rolipram at concentrations up to 10 μM. These results are at variance with another study performed with the TPA-Mat human T-cell line, where a range of cyclic AMP-elevating agents including the non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), markedly attenuated PMA-induced proliferation (Goto *et al.*, 1988). The reason for this inconsistency is unclear but may simply reflect a fundamental difference in the regulation of the mitogenic response between TPA-Mat cells and $CD4^+$ and $CD8^+$ T-lymphocytes. Since elevation of the cytosolic free Ca^{2+} concentration and activation of one or more PKC isoenzymes are intimately involved in TCR-mediated proliferation, the data described in this study clearly indicate that cyclic AMP

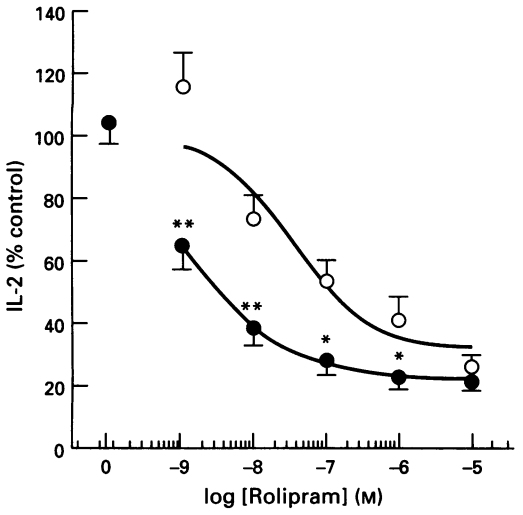

Figure 12 Effect of SK&F 95654 on the ability of rolipram to suppress PHA-induced IL-2 generation by human peripheral blood $CD4^+$ T-lymphocytes. Highly purified $CD4^+$ T-cells were cultured with rolipram (100 nM to 10 μM) in the absence (○) or presence (●) of SK&F 95654 (10 μM) in supplemented RPMI 1640 and exposed to PHA (5 $\mu g ml^{-1}$). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of IL-2 released into the culture medium was measured at 16 h by a specific ELISA. Data represent the mean of seven independent experiments conducted with different cell preparations; vertical lines show s.e.mean. See Methods for further details. *$P<0.05$ – significant potentiation of the inhibitory effect induced by rolipram.

inhibits this response by interacting at, or proximal to, the level of PtdIns(4,5)P_2 hydrolysis. Although not formally investigated in this study, possible phosphorylation sites at which cyclic AMP-dependent protein kinase could suppress mitogenesis include PLCγ1, at serine 1248 (Park *et al.*, 1992), and as yet unidentified serine/threonine residues in the γ and ε polypeptides that are part of the TCR complex (Klausner *et al.*, 1987; Patel *et al.*, 1987).

An unexpected finding from the studies with PMA and ionomycin was that although rolipram (10 μM) failed to inhibit proliferation, it markedly suppressed (by between 55 and 80%) the elaboration of IL-2 (and IFNγ). These data are difficult to interpret given that T-cell proliferation is believed to be governed solely by IL-2; however, at least two explanations are worthy of consideration. Perhaps the most simple possibility is that IL-2 synthesis needs to be almost completely inhibited or even abolished before an anti-mitogenic effect of rolipram is observed. In this scenario, any residual IL-2 produced by the co-addition of PMA and ionomycin in rolipram-treated cells would still be sufficient to drive proliferation via the high affinity IL-2R. This interpretation, however, is difficult to reconcile with the anti-proliferative action of rolipram when PHA and anti-CD3 are used as mitogens at concentrations that stimulated the uptake of an equivalent amount of [^{3}H]-thymidine as PMA/ionomycin. Alternatively, a more heretical explanation is that proliferation of T-lymphocytes is also regulated by an IL-2-independent mechanism(s). Support for this contention is the finding that rolipram was ~60-times more potent at suppressing PHA-induced IL-2 generation (IC_{50} ~180 nM) from $CD4^+$ T-lymphocytes than at inhibiting the cellular incorporation of [^{3}H]-thymidine (IC_{50} ~11 μM). Although not formally investigated in this study possible candidates include IL-15 which shares many of the biological actions of IL-2 and is abundantly expressed by mononuclear cells (Giri *et al.*, 1995). Indeed, IL-15 is a chemoattractant for T-lymphocytes (Wilkinson & Liew, 1995) and promotes cell growth and division (Lewko *et al.*, 1995; Seder *et al.*, 1995).

In conclusion, the results of these studies demonstrate that human peripheral blood $CD4^+$ and $CD8^+$ T-lymphocytes express representatives of the PDE3 and PDE4 isoenzyme families that are differentially distributed between the particulate and soluble fractions, respectively. Furthermore, studies with selective inhibitors demonstrate a functional role for these isoenzymes in regulating cell growth, division and cytokine biosynthesis. Finally, these data provide the first evidence that human T-lymphocytes have the potential to express the recently identified PDE7. Analysis of the functional role of this enzyme, however, must await the development of selective inhibitors.

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