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Evidence that cyclic AMP phosphodiesterase inhibitors suppress interleukin-2 release from murine splenocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer

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1 We have investigated the suppressive effects of rolipram, RP 73401 (piclamilast) and other structurally diverse inhibitors of cyclic AMP-specific phosphodiesterase 4 (PDE4) on interleukin (IL)-2 generation from Balb/c mouse splenocytes exposed to the superantigen, *Staphylococcocal* enterotoxin-A (*Staph.* A). The purpose was to determine whether their potencies are more closely correlated with inhibition of PDE4 from CTLL cells, against which rolipram displays weak potency (low-affinity PDE4), or displacement of [^{3}H]-(±)-rolipram from its high-affinity binding site (HARBS) in mouse brain cytosol.

2 RP 73401 (IC_{50} 0.46±0.07 nM, $n=4$) was a very potent inhibitor of *Staph.* A-induced IL-2 release from Balb/c mouse splenocytes, being >1100 fold more potent than (±)-rolipram (IC_{50} 540±67 nM, $n=3$).

3 A close correlation ($r=0.95$) was observed between suppression of IL-2 release by PDE inhibitors and inhibition of PDE4. In contrast, little correlation ($r=0.39$) was observed between suppression of IL-2 release and their affinities for the high-affinity rolipram binding site (HARBS).

4 RP 73401 only inhibited partially (30–40%) *Staph.* A-induced incorporation of [^{3}H]-thymidine into splenocyte DNA. The PDE3 inhibitor, siguazodan (10 μM), had little or no effect on IL-2 release or DNA synthesis. This concentration of siguazodan did not enhance the inhibitory action of RP 73401 on IL-2 release but potentiated its effect on DNA synthesis, increasing potency and efficacy.

5 *Staph.* A-induced DNA synthesis was only partially inhibited by anti-IL-2 neutralizing antibody, whereas dexamethazone (100 nM) and cyclosporine A (100 nM) completely blocked the response.

6 RP 73401 (IC_{50} 6.3±1.9 nM, $n=4$) was 140 fold more potent than rolipram (IC_{50} 900±300 nM, $n=3$) in inhibiting *Staph.* A-induced [^{3}H]-thymidine incorporation into splenocyte DNA.

7 The results implicate a low-affinity form of PDE4 in the suppression of *Staph.* A-induced IL-2 release from murine splenocytes by PDE inhibitors. The data also indicate that mitogenic factors other than IL-2, whose elaboration or responses to which are regulated by PDE3 as well as PDE4, contribute to the superantigen-induced DNA synthesis.

Keywords: Cyclic AMP-phosphodiesterase; RP 73401; rolipram; interleukin-2; murine splenocytes; *Staphylococcal* enterotoxin A; DNA synthesis

Introduction

The therapeutic potential of adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-specific phosphodiesterase 4 (PDE4) inhibitors has attracted considerable attention, primarily because of their abilities to dampen functions of several inflammatory and immunocompetent cells implicated in chronic inflammatory diseases (Palfreyman & Souness, 1996). Most interest to date has focused on the possible anti-asthma effects of these compounds but, more recently, observation of the potent suppression of tumour necrosis factor-α (TNFα) production from mononuclear phagocytes (Schade & Schudt, 1993; Semmler *et al*., 1993; Prabhakar *et al*., 1994) by PDE4 inhibitors suggests their potential utility in treating autoimmune diseases associated with over-elaboration of this pro-inflammatory cytokine, such as arthritis and multiple sclerosis (Sekut *et al*., 1995; Genain *et al*., 1995; Sommer *et al*., 1995). Dual neutralization of TNFα and T-cell functions with TNFα receptor IgG fusion protein in combination with anti-CD-4 antibody results in a greater therapeutic effect in animal models or arthritis than the targeting of TNFα alone (Williams *et al*., 1995). It is of interest, therefore, that cyclic AMP PDE inhibitors also suppress T-lymphocyte proliferative responses to a number of stimuli and inhibit the release of the important T-cell mitogen, interleukin (IL)-2 (Averill *et al*., 1988; Robiscek *et al*., 1989; Lewis *et al*., 1993).

Large numbers of compounds of diverse structures have been synthesized which are believed to exert their anti-inflammatory effects through inhibition of PDE4 (Palfreyman & Souness, 1996; De Brito *et al*., 1997). However, in some instances, poor correlations have been obtained 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; Barnette *et al*., 1995a; 1996; Kelly *et al*., 1996) of PDE inhibitors are more tightly correlated with their potencies in displacing [^{3}H]-rolipram from brain membranes than inhibition of PDE4. Indeed, evidence has emerged recently suggesting that this is the cause of the PDE4-elicited emesis in dogs and ferrets (Barnette *et al*., 1995b; Duplantier *et al*., 1996). Although this binding site 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 greater than its apparent antagonist binding affinity ($K_{i,app}$) in the binding assay (Torphy *et al*., 1992). Furthermore, whereas the **R**-(−)-enantiomer of roli-

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pram 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).

Although the nature of the high-affinity binding site for rolipram (HARBS) has not been proved unambiguously, compelling evidence points to it representing a distinct conformer of PDE4 with which rolipram interacts with high affinity (HA-PDE4) (Christensen *et al*., 1996; Jacobitz *et al*., 1996). There is evidence for a different cell/tissue distribution of HA-PDE4 and the form with which rolipram interacts with relatively low-affinity (>100 nM; LA-PDE4) (Souness & Rao, 1997). High levels of HARBS have been measured in brain (Schneider *et al*., 1986) and, although difficult to detect in peripheral tissues, its presence has been demonstrated in cell types (parietal cells, macrophages) in which functional responses to PDE inhibitors are better correlated with displacement of [^{3}H]-rolipram from HARBS than inhibition of PDE4 catalytic activity (Barnette *et al*., 1995a; Kelly *et al*., 1996). The factors that determine the conformation of PDE4 are uncertain although it is known that phosphorylation (RNPDE4D3) (Sette *et al*., 1994) and membrane association (HSPDE4A) (Huston *et al*., 1997) of some PDE4 subtype splice variants have marked pharmacological effects.

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 responses of murine splenocytes to *Staphylococcal* enterotoxin A (*Staph*. A), a superantigen which activates T-cells without being processed by antigen presenting cells through binding to MHC II and the V_β-chain of the T-cell receptor (Fischer *et al*., 1989; Fraser, 1989). Studies have been performed to determine whether their suppression of interleukin (IL)-2 generation is more closely associated with inhibition of PDE4 catalytic activity from murine CTLL lymphocytes, against which rolipram exhibits relatively weak potency (low-affinity (LA)-PDE4), or displacements of [^{3}H]-(±)-rolipram from its high-affinity binding site (HARBS). The results demonstrate that PDE inhibitor actions on splenocyte IL-2 release are better correlated with PDE4 inhibition than with displacement of [^{3}H]-rolipram from HARBS suggesting that, as in monocytes (Souness *et al*., 1996; Barnette *et al*., 1996), PDE4 exists predominantly in a 'low-affinity' state in spleen-derived T-lymphocytes. Evidence is also presented demonstrating that cyclic AMP PDE inhibitors suppress *Staph*. A-induced splenocyte DNA synthesis by acting on processes additional to IL-2 release.

Methods

Preparation of splenocytes

Spleens were removed from naive Balb/c mice, placed in RPMI 1640 medium with Glutamax 1 and HEPES, 25 mM (Gibco Brl), containing foetal calf serum (10%), penicillin/streptomycin (100 u ml^{-1}) and β-mercaptoethanol (50 μM) and pressed through a 70 micron cell strainer (Falcon) into a plastic tube (50 ml). The splenocytes were centrifuged (1000 *g*, 10 min) and resuspended in erythrocyte lysing buffer (NH_4Cl 0.154 mM, $KHCO_3$ 0.012 mM and ethylenediaminetetraacetic acid (EDTA) 0.1 mM). After 10 min, the cells were centrifuged (1000 *g*, 10 min), resuspended in RPMI medium and cultured overnight (37°C, 5% CO_2/95% O_2). The cells were then washed (×3) with RPMI medium, resuspended and counted (haemocytometer). Viability was determined by Trypan blue exclusion.

Cell incubations and IL-2 determinations

Cells were aliquoted into 96 well microtitre plates at a density of 2×10^6 ml^{-1} and incubated for 48 h with *Staph*. A (100 ng ml^{-1}) plus compounds or vehicle. At the end of the incubation, plates were centrifuged (500 *g*, 10 min) and 150 μl of the supernatant were transferred to rat anti-mouse IL-2 capture antibody-coated 96-well microtitre plates. After the plates had been washed (150 μl PBS/Tween-20, ×4), the unoccupied sites blocked with 150 μl blocking buffer (bovine serum albumin 1%, Na_2CO_3 7 mM, $NaHCO_3$ 17 mM) and rewashed (150 μl PBS/Tween-20 ×4), IL-2 was measured by sandwich ELISA, by use of a biotinylated rat anti-mouse IL-2 monoclonal detection antibody and streptavidin-peroxidase conjugate. The peroxidase substrate was 3,3′,5′,5′-tetramethylbenzidine (TMB), in the presence of hydrogen peroxide. Absorbance at 450 nm was measured with a Titertek Multiscan plate reader. Recombinant mouse IL-2 was used as the standard in the ELISA assay.

Measurement of [^{3}H]-thymidine incorporation into splenocytes

Naive splenocytes, cultured overnight before use, were dispensed into 96 well microtitre plates (3.2×10^5 cells 160 μl^{-1}) as in the IL-2 release experiments. Compounds (20 μl in culture medium) followed by 20 μl of *Staph*. A (100 ng ml^{-1}) were then added and the cells were cultured for 24 h. [^{3}H]-methylthymidine (1 μCi, 20 μl) was then added to each well and, after 18 h, the cells were collected on membrane filters by use of a Packard Filtermate 196 Cell Harvester. [^{3}H]-thymidine incorporation into the cells was measured with a Packard Topcount Microplate Liquid Scintillation Counter.

Culture of murine CTLL lymphocytes and preparation of subcellular fractions

CTLL cells, an IL-2-dependent lymphocytic cell line derived from a C57b1/6 mouse, were obtained from the European Collection of Animal Cell Culture Catalogue (ECACC). Cells were grown in suspension (37°C, 5% CO_2) in RPMI-Glutamax I medium (Gibco) supplemented with 5% foetal calf serum, penicillin (50 u ml^{-1})/streptomycin (50 μg ml^{-1}), β-mercaptoethanol (50 μM) and IL-2 (10 ng ml^{-1}). Cells (10^8) were centrifuged (250 *g*, 10 min) and washed twice with 50 ml phosphate-buffered saline (PBS). The cell pellet was resuspended in ice-cold homogenization buffer (Tris/HCl 20 mM (pH 7.5), $MgCl_2$ 2 mM, dithiothreitol 1 mM, EDTA 5 mM, Triton X-100 0.1%, sucrose 0.25 M, *p*-tosyl-L-lycine-chloromethylketone (TLCK) 10 μM, leupeptin 10 μg ml^{-1} and aprotinin 2000 u ml^{-1}) and homogenized with a Dounce homogenizer (20 strokes). The homogenate was used in PDE assays.

Preparation of human peripheral blood monocytes

The isolation of human monocytes and the preparation of subcellular fractions is as described in Souness *et al*. (1996).

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} and [^{3}H]-cyclic AMP 1 μM (100000 c.p.m./assay). Unless stated otherwise, the substrate concentration was 1 μM. Assays were performed in the presence of siguazodan to inhibit the PDE3 activity present in CTLL cells.

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.

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 subtypes referred to in this paper are based on human cDNA nucleotide sequences (PDE4A-D) as presented by Bolger (1994) and McLaughlin *et al*. (1993).

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

Brains from Balb/c mice were homogenized in homogenization buffer (Tris-HCl 50 mM (pH 7.4), $MgCl_2$ 5 mM, dithiothreitol 0.1 mM TLCK, 20 μM) and cytosolic and membrane fractions prepared (30000 *g*, 30 min – High-speed 65 M Europa Ultracentrifuge). The binding assay was performed on 24 well plate GF/B Unifilter plates either untreated (membrane assay) or soaked in 0.3% polyethylenimine (PEI) (cytosolic assay) by use of a Packard Harvester Filtermate 196 fitted with a Unifilter-24 probed head (Canberra Packard), essentially as described previously by Schneider *et al*. (1986) with [^{3}H]-rolipram (2 nM) and cytosolic or membrane samples corresponding to 500 μg of original brain tissue.

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-cyclopentyloxy-4-methoxybenzamide) were synthesized by the department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd (Dagenham, Essex, U.K.). Ibudilast (3-isobutyryl-2-isopropylpyrazolo [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-pyrimidol[6,1-a]isoquinolin-4-one, HL-725) was purchased from Calbiochem-Novabiochem U.K. Ltd (Nottingham, U.K.). Rat anti-mouse IL-2 capture monoclonal antibody, biotinylated rat anti-mouse IL-2 detection monoclonal antibody and rat anti-mouse IL-2 neutralizing monoclonal antibody were from Pharmingen (Cambridge Bioscience, Cambridge, U.K.). Recombinant mouse IL-2 was from Genzyme Diagnostics (Cambridge, MA, U.S.A.). 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}) and cyclic [2,8-^{3}H]-AMP (41 Ci mmol^{-1}) were from Amersham International (Amersham, Bucks., U.K.). 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 24 and 96-well filtration (0.2 μm) plates from Millipore, U.K. Ltd (Harrow, U.K.). 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

Data are presented as means ± s.e.mean and analysed by Student's *t* test. Values were considered to be statistically significant when P was less than 0.05. To investigate whether statistically significant ($P<0.05$) relationships existed between inhibition of IL-2 release 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 ρ) of the respective $\log_{10}$M values were conducted by use of the RS/1 programme (BBN Software Products Corporation, Cambridge, MA, U.S.A.).

Results

Inhibition of IL-2 generation

Staph. A was a powerful stimulus for the elaboration of IL-2 from splenocytes with 50–80 u ml^{-1} being routinely detected in media at the end of experiments following exposure of cells to the superantigen in the absence of other additions. RP 73401 (IC_{50} 0.46 ± 0.07 nM, $n=4$) was a very potent inhibitor of *Staph.* A-induced IL-2 release, being >1100 fold more potent than rolipram (IC_{50} 540 ± 67 nM, $n=3$) (Figure 1a;

Table 1 Potencies of PDE4 inhibitors in inhibiting CTLL cell PDE4, displacing [^{3}H]-rolipram binding and suppressing splenocyte IL-2 release

Compound	*PDE4 inhibition* (IC_{50} μM)	*Displacement of [^{3}H]-rolipram* ($K_{i_{app.}}$ μM) *Cytosolic*	*Displacement of [^{3}H]-rolipram* ($K_{i_{app.}}$ μM) *Particulate*	*Suppression of IL-2 release* (IC_{50} μM)
RP 73401	0.0011	0.0008	0.0013	0.00046
R-(−)-rolipram	0.30	0.0007	0.001	0.33
S-(+)rolipram	1.3	0.0077	0.012	3.0
(±)-Rolipram	0.64	0.0016	0.0023	0.54
Denbufylline	1.2	0.0032	0.0036	0.90
Ro 20-1724	4.0	0.016	0.024	0.44
Ibudilast	1.1	0.0046	0.011	1.1
IBMX	11	0.54	1.1	28
Trequinsin	0.52	2.7	3.0	0.37
Compound A	0.0057	ND	ND	0.00052
Compound B	0.0034	0.0048	0.0064	0.0050
Compound C	0.0014	0.0031	0.0029	0.0038
Compound D	0.014	0.015	0.014	0.0072

Cyclic AMP PDE activity was measured in homogenates of murine CTLL cells in the presence of siguazodan (10 μM). [^{3}H]-rolipram binding was measured in the cytosolic and membrane fractions of mouse brain. IL-2 release from murine splenocytes was stimulated with *Staph.*A (100 ng ml^{-1}). The results represent the means of 2–4 experiments.

Table 1). **R**-(−)-rolipram (IC_{50} 333 ± 38 nM, $n=3$) was 7.9 fold more potent than **S**-(+)-rolipram (IC_{50} 3000 ± 320 nM, $n=3$) in inhibiting the *Staph* A-induced response (Figure 1b). The potency difference between RP 73401 was similar to that observed for inhibition of CTLL cell PDE4 (a convenient source of LA-PDE4) (640 fold) but contrasts markedly with potencies for displacement of [^{3}H]-rolipram from HARBS against which the two compounds were equipotent (Table 1). Similarly, trequinsin was equipotent with rolipram in inhibiting PDE4 catalytic activity and IL-2 release but was over 300 fold less potent in competing with rolipram for HARBS (Table 1). The rolipram enantiomeric potency difference for IL-2 suppression was intermediate between CTLL cell PDE4 inhibition (4.3 fold difference) and displacement of [^{3}H]-rolipram from mouse brain cytosol (11 fold difference) (Table 1, Figure 1b).

Studies were performed to determine whether inhibition of IL-2 release by a range of structurally distinct PDE inhibitors is better correlated with inhibition of CTLL cell PDE4 or displacement of [^{3}H]-rolipram from HARBS in the cytosolic fraction of mouse brain. There was a close correlation ($r=0.96$, $P<0.01$, $n=12$; Spearman's $\rho=0.85$, $P<0.01$, $n=12$) between inhibitory potencies against the CTLL-cell PDE4 and enzyme from human monocytes (Souness *et al*., 1996) (Table 1, Figure 2a). Furthermore, the fact that the IC_{50} values of rolipram, against both enzymes was greater than 200 nM indicates that both are 'low-affinity' conformers (LA-PDE4) (Souness *et al*., 1996; Jacobitz *et al*., 1996). It should be noted that there was a poor correlation ($r=0.42$; Spearman's $\rho=0.38$) between inhibition of CTLL-cell PDE4 and displacement of [^{3}H]-rolipram from HARBS (Table 1, Figure 2b).

A close correlation ($r=0.95$, $P<0.01$, $n=12$; Spearman's $\rho=0.91$, $P<0.01$, $n=12$) existed between suppression of IL-2 release by PDE inhibitors and inhibition of CTLL cell PDE4 (Table 1, Figure 3a). In contrast, a poor correlation ($r=0.37$; Spearman's $\rho=0.39$) was observed between suppression of IL-2 release and displacement of [^{3}H]-(±)-rolipram from its binding site in brain cytosol (Table 1, Figure 3b).

Zaprinast, a selective PDE5 inhibitor had little effect on *Staph*. A-induced IL-2 release at concentrations up to 100 μM (9.5 ± 1.5% inhibition at 100 μM, $n=3$). The PDE3 inhibitor, siguazodan, alone, had little effect on *Staph*. A-induced IL-2 release (25 ± 1% inhibition, $n=3$, at 100 μM) and a submaximal concentration of siguazodan (10 μM) failed to potentiate the suppressive effects of RP 73401 (Figure 4). Prostaglandin E_2 (10 μM) which stimulates cyclic AMP accumulation through activation of adenylyl cyclase, also failed to

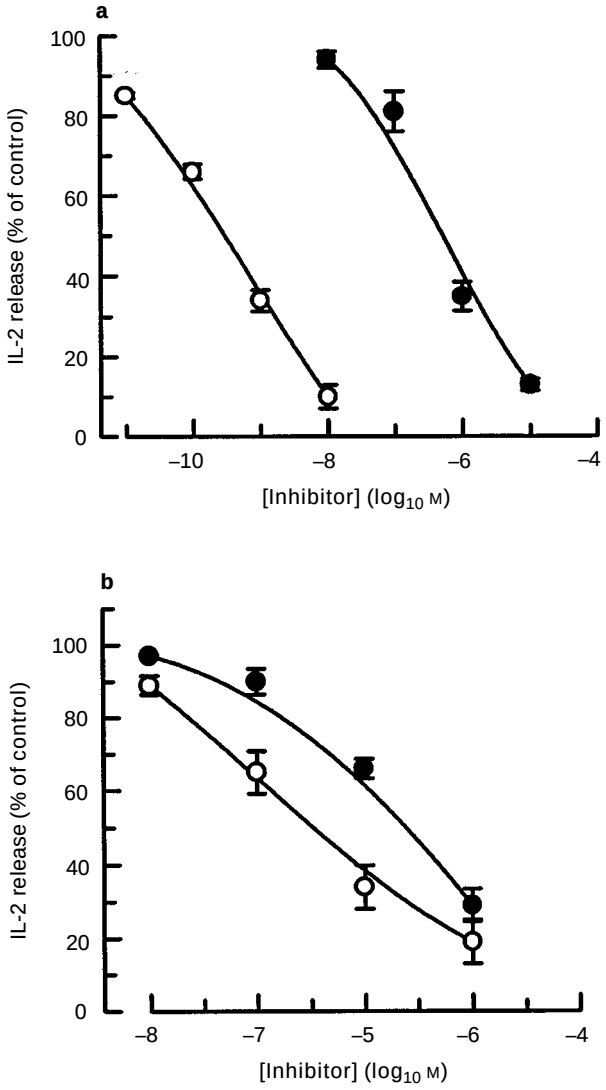

Figure 1 Inhibition of *Staph*. A-induced interleukin-2 (IL-2) release by (a) RP 73401 (○) and (±)-rolipram (●) and (b) **R**-(−)-rolipram (○) and **S**-(+) rolipram (●). Data show means and vertical lines indicate s.e.mean.

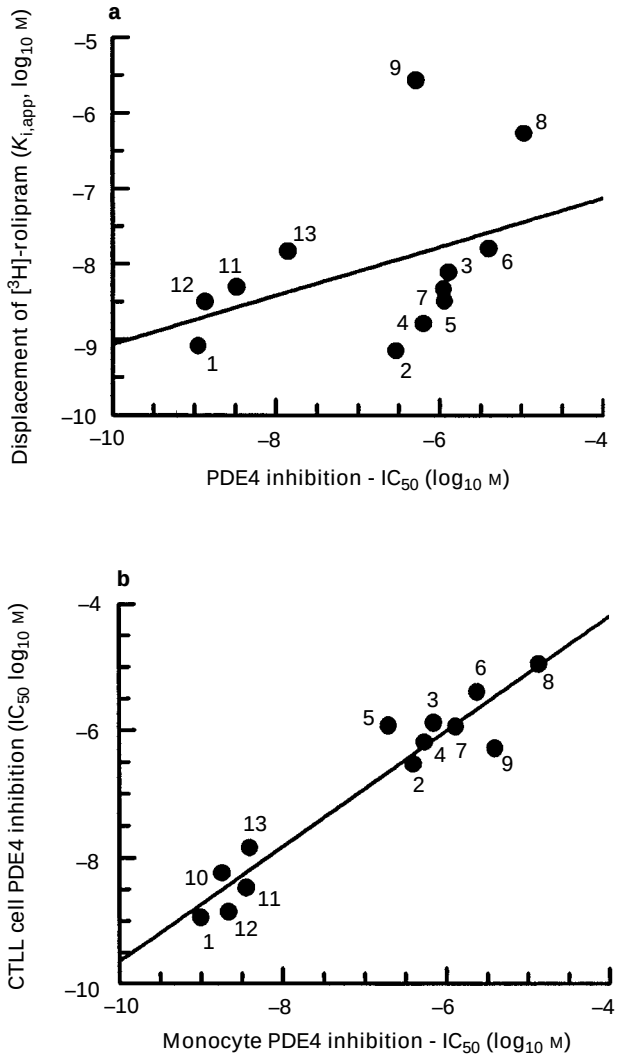

Figure 2 Inhibition of CTLL cell PDE4 as a function of displacement of [^{3}H]-rolipram binding to murine brain membranes (a) and inhibition of human monocyte PDE4 (b). Rolipram binding data are expressed as $K_{i,app}$ values (log_{10}M) and PDE4 data as IC_{50} values (log_{10}M). Compounds: (1) RP 73401; (2) **R**-(−)-rolipram; (3) **S**-(+)-rolipram; (4) (±)-rolipram; (5) denbufylline; (6) Ro 20-1724; (7) ibudilast; (8) IBMX; (9) trequinsin; (10) compound A; (11) compound B; (12) compound C; (13) compound D.

potentiate the action of RP 73401 (data not shown). Dexamethazone (1 μM) inhibited *Staph*. A-induced IL-2 generation by approximately 85%.

Inhibition of DNA synthesis

Because previous studies have demonstrated that PDE4 inhibitors act synergistically with PDE3 inhibitors in inhibiting mitogen-induced T-lymphocyte proliferation (Lewis *et al*., 1993), we investigated the effects of RP 73401, alone, and in combination with siguazodan (10 μM), on *Staph*. A-induced incorporation of [^{3}H]-thymidine into murine splenocyte DNA. As shown in Figure 5, RP 73401 elicited an incomplete (30–40% at 100 nM) inhibition of the response to this mitogen. Siguazodan (10 μM), which, alone, was without effect, markedly potentiated the suppression of DNA synthesis by RP 73401 (Figure 5). RP 73401 (IC_{50} 6.3$\pm$1.9 nM, $n=4$) was 140 fold more potent than rolipram (IC_{50} 900$\pm$300 nM, $n=3$) against *Staph*. A-induced [^{3}H]-thymidine incorporation into splenocyte DNA (Figure 6). Dexamethazone (100 nM) and cyclosporine A (100 nM, inhibited DNA synthesis in response to *Staph*. A by greater than 90%. Similar results were observed when concanavalin A was employed as the mitogenic stimulus (data not shown).

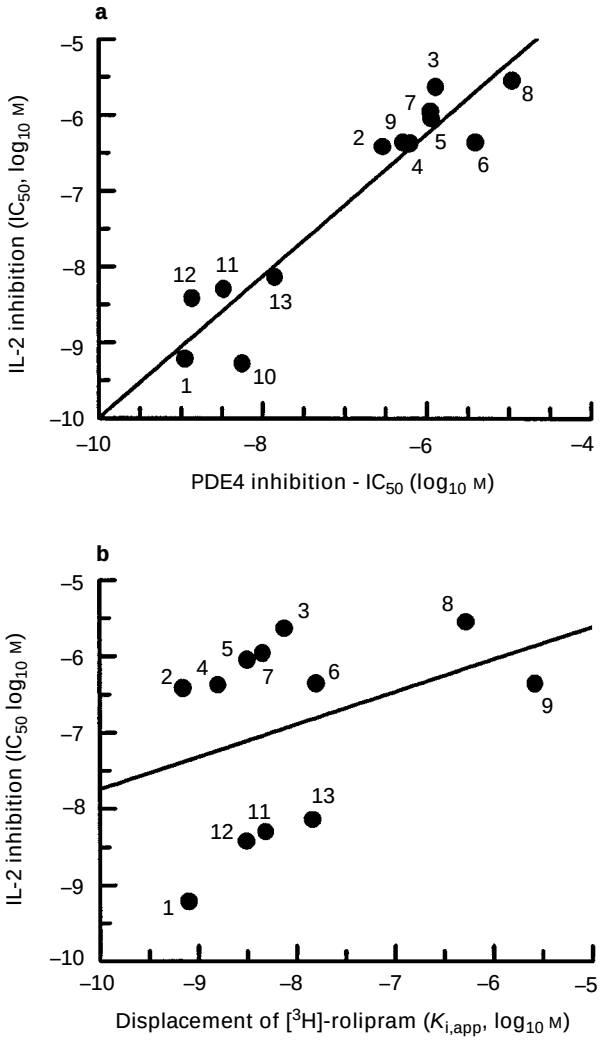

Figure 3 Inhibition of *Staph*. A-induced interleukin (IL-2) release as functions of inhibition of CTLL cell PDE4 (a) and displacement of [^{3}H]-rolipram binding to the cytosolic fraction of murine brain (b). Rolipram binding data are expressed as $K_{i,app}$ values ($\log_{10}$M), PDE4 data as IC_{50} values ($\log_{10}$M) and IL-2 data as IC_{50} values ($\log_{10}$M). Compounds: 1–13 are as in the legend to Figure 2.

To determine the importance of IL-2 in *Staph*. A-induced DNA synthesis, the effects of a neutralizing antibody to IL-2 were studied. Anti-IL-2 antibody only partially inhibited the incorporation of [^{3}H]-thymidine stimulated by the superantigen. Compared to incubations performed in the presence of non-specific mouse IgG (10 μM ml^{-1}), anti-IL-2 antibody (10 μg ml^{-1}) only reduced [^{3}H]-thymidine incorporation by 16%. Incomplete inhibition of concanavalin A-induced DNA synthesis was also observed. In contrast, an identical concentration of the antibody completely blocked the proliferative response to IL-2 in the IL-2 responsive cell-line, CTLL-2 (data not shown).

Discussion

As discussed in the Introduction, several functional effects of PDE4 inhibitors including those which have hindered their development as potentially important anti-inflammatory

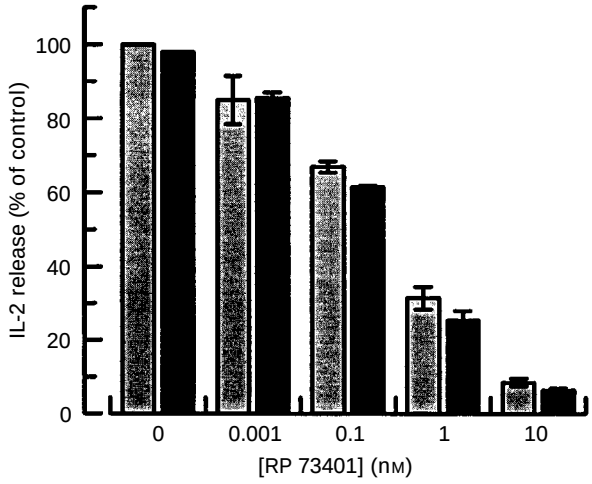

Figure 4 Lack of potentiation by siguazodan of RP 73401 inhibition of *Staph*. A-induced interleukin-2 (IL-2) release. Cells were stimulated with *Staph*. A in the presence of RP 73401 alone (stippled columns) or RP 73401 plus siguazodan (10 μM) (solid columns) as described in the Methods section. The results represent the means$\pm$ s.e.mean of 3 separate experiments.

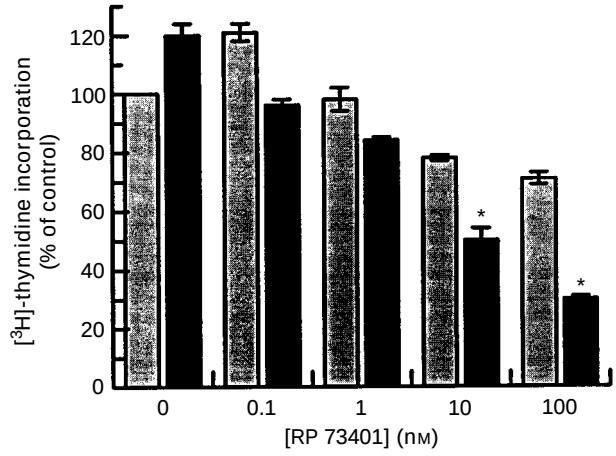

Figure 5 Potentiation by siguazodan of RP 73401 inhibition of *Staph*. A-induced [^{3}H]-thymidine incorporation into murine splenocytes. Cells were stimulated with *Staph*. A in the presence of RP 73401 open (stippled columns) or RP 73401 plus siguazodan (10 μM) (solid columns) as described in the Methods section. The results represent the means$\pm$s.e. mean of 3 separate experiments. *$P<0.01$ for significant difference between RP 73401 alone and in the presence of siguazodan.

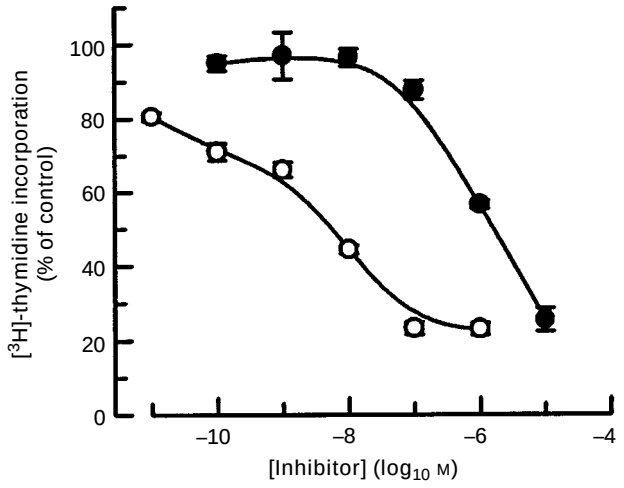

Figure 6 Comparison of the potencies of RP 73401 and rolipram in inhibiting *Staph.* A-induced [^{3}H]-thymidine incorporation into murine splenocytes in the presence of siguazodan. Cells were stimulated with *Staph.* A with increasing concentrations of RP 73401 (○) or rolipram (●) in the presence of siguazodan (10 μM). The results represent the means of 3 separate experiments; vertical lines show s.e.mean.

agents are more closely associated with their affinities for HARBS (HA-PDE4) than inhibition of PDE4 catalytic activity (LA-PDE4) (Harris *et al*., 1989; Schmiechen *et al*., 1990; Souness & Scott, 1993; Barnette *et al*., 1995a,b; 1996; Kelly *et al*., 1996; Duplantier *et al*., 1996). However, it is becoming clear that some potentially beneficial functional effects of PDE inhibitors are poorly correlated to the potencies of these compounds in binding to HARBS. Recently, two independent studies demonstrated that there is a better association between suppression of TNFα release from human monocytes and inhibition of LA-PDE4 catalytic activity than competition with [^{3}H]-rolipram for HARBS (Souness *et al*., 1996; Barnette *et al*., 1996). We have now extended this further in demonstrating that another effect of PDE4 inhibitors of clinical relevance, namely suppression of the release of the important T-cell mitogen IL-2 from splenocytes, is also closely associated with inhibition of PDE4. The lack of functional importance of HARBS in modulating IL-2 release is exemplified by the more than 1000 fold greater potency of RP 73401, a compound that has been proposed not to discriminate between different PDE4 conformers (Souness & Rao, 1997), compared to the HA-PDE4-selective rolipram. A greater than 600 fold difference in potency was also observed against PDE4 from CTLL cells, whereas the compounds were equipotent against HARBS. This potency difference between RP 73401 and rolipram contrasts sharply with their actions in cells and tissues in which functional effects of PDE4 inhibitors are closely associated with their affinities for HARBS (Harris *et al*., 1989; Souness *et al*., 1995). For example, RP 73401 is only 3–4 fold more potent than rolipram in inhibiting methacholine-induced contraction of guinea-pig trachealis and potentiating isoprenaline-induced cyclic AMP accumulation in guinea-pig peritoneal eosinophils (Souness *et al*., 1995). Furthermore, trequinsin, which interacts weakly with HARBS and is 100 fold less potent than rolipram in inducing cyclic AMP accumulation in eosinophils (Souness *et al*., 1991; Souness & Scott, 1993), is equipotent with the archtypal PDE4 inhibitor in suppressing splenocyte IL-2 release. Thus, these results support the concept of pharmacologically distinct PDE4 forms being differentially distributed between cells and offer hope that if selective inhibitors of the 'low-affinity' PDE4 can be identified they will suppress the functions of at least two cell-types (monocytes, T-cells) implicated in chronic inflammatory diseases.

The failure of siguazodan, to influence the potent inhibitory effect of RP 73401 on *Staph.* A-induced IL-2 release from murine splenocytes contrasts with recent studies on human peripheral blood $CD4^+$ and $CD8^+$T-cells in which the PDE3 inhibitor, SK&F 95654, clearly potentiated rolipram suppression of phytohaemagglutinin (PHA)- and anti-CD3-induced IL-2 generation (Giembycz *et al*., 1996). The reason(s) for this discrepancy is/are uncertain although distinct PDE profiles in IL-2 generating T-cells from blood and spleen and the use of different stimuli, the prolongued exposure to which may affect PDE profiles, offer possible explanations. In contrast to IL-2 release, [^{3}H]-thymidine incorporation into splenocytes in response to the T-cell mitogen, *Staph.* A, was only partially inhibited by RP 73401 and this effect was markedly potentiated by siguazodan, which alone only exerted a weak effect. Dual actions of PDE4 and PDE3 inhibitors on T-cell proliferation have been found previously in a mixed population of purified, human T-lymphocytes (Robicsek *et al*., 1991), highly purified preparations of human CD4+ and CD8+T-cells (Giembycz *et al*., 1996) and in murine splenocytes (Lewis *et al*., 1993).

Previous studies also support a dissociation between suppression of IL-2 and the anti-proliferative effects of cyclic AMP PDE inhibitors (Lewis *et al*, 1993; Giembycz *et al*., 1996). For example, although rolipram suppresses IL-2 generation in human $CD4^+/CD8^+$ lymphocytes in response to a combination of phorbol myristate acetate (PMA) and ionomycin, it does not inhibit the proliferative response to the same stimuli (Giembycz *et al*., 1996). Such discrepancies between cytokine and proliferation data indicate either that a low concentration of IL-2, whose release is resistant to PDE4 inhibitors, alone, is fully capable of stimulating proliferation or that the release of, or responses to, T-cell mitogens other than IL-2 are sensitive to inhibition of PDE4 and PDE3. The fact that [^{3}H]-thymidine incorporation into splenocyte DNA was only partially inhibited by a blocking anti-IL-2 antibody strongly supports the latter explanation. These and previous (Giembycz *et al*., 1996) results on the effects of PDE4 inhibitor actions on T-cells bring into question the previously held paradigm of IL-2, which is released following T-cell receptor activation, being the principal mitogen in pushing T-cells through from the G_0 to the G_1 stage of the cell cycle (Theze *et al*., 1996). The identity of the putative, alternative T-cell mitogen(s) in splenocyte preparations is not known with certainty, although IL-15 is a strong candidate. This cytokine is a T-cell growth factor expressed by mononuclear cells that shares activities and receptor components with IL-2 (Giri *et al*., 1995). Several studies indicate that PDE inhibitors may exert their anti-proliferative effects by blocking mitogenic signal transduction systems (van Tits *et al*., 1991; Anastassiou *et al*., 1992). Indeed, agents that elevate cyclic AMP inhibit anti-CD3-induced tyrosine phosphorylation of a 100 kDa protein implicated in T-cell activation as well as decreasing IL-2 biosynthesis and IL-2 receptor expression (Anastassiou *et al*., 1992). These, and the data presented in this paper suggest that PDE inhibitors may have multiple inhibitory effects on T-cell proliferation. It is of interest that, although inhibition of DNA synthesis and IL-2 release by PDE inhibitors appeared to be poorly related, the much greater potency of RP 73401 compared to rolipram in suppressing *Staph.* A-induced [^{3}H]-thymidine incorporation into splenocytes suggests that this effect, like IL-2 suppression is linked to a 'low-affinity' form of PDE4.

In summary, the results demonstrate that suppression of *Staph.* A-induced IL-2 release from murine splenocytes is better correlated with inhibition of cyclic AMP hydrolysis by CTLL cell lysates than displacement of [^{3}H]-rolipram from its high-affinity binding site, implicating a low-affinity form of PDE4 in the regulation of this response. Although also probably the case for inhibition of splenocyte DNA synthesis, this effect of PDE4 inhibitors is largely independent of IL-2. If selective inhibitors of LA-PDE4 can be discovered, then their abilities to inhibit both TNFα and IL-2 release could hold great promise for the treatment of inflammatory conditions such as arthritis.

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