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Suppression of Anti-CD3-Induced Interleukin-4 and Interleukin-5 Release from Splenocytes of *Mesocestoides Corti*-Infected BALB/c Mice by Phosphodiesterase 4 Inhibitors

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ABSTRACT. We investigated the suppressive effects of rolipram, RP 73401 (piclamilast), and other structurally diverse inhibitors of adenosine 3′5′-cyclic monophosphate (cAMP)-specific phosphodiesterase (PDE4) on anti-CD3-stimulated interleukin (IL)-4 and IL-5 generation by splenocytes from BALB/c mice infected with *Mesocestoides* (M) *corti*. RP 73401 (IC_{40}: 0.011 ± 0.004 μM) was a very potent inhibitor of anti-CD3-induced IL-4 release, being ~40-fold more potent than (±)-rolipram (IC_{40}: 0.43 ± 0.09 μM). A maximal inhibition of 60–70% of the response was achieved at the top concentrations of RP 73401 (1 μM) and rolipram (100 μM). These PDE inhibitors also suppressed IL-5 generation over the same concentration ranges, but the maximal suppression achieved was only 30–40%. *R*-(−)-rolipram (IC_{40}: 0.39 ± 0.09 μM) was ~6-fold more potent than *S*-(+)- rolipram (IC_{40}: 2.6 ± 0.95 μM) in inhibiting IL-4 release. A close correlation (r^2 = 0.82) was observed between suppression of IL-4 release by PDE inhibitors and inhibition of CTLL cell PDE4, a form against which *R*-(−)-rolipram displayed relatively weak inhibitory potency. A poorer correlation (r^2 = 0.26) was observed between suppression of IL-4 release and affinities of cAMP PDE inhibitors for the high-affinity rolipram binding site in mouse brain membranes. The cGMP-inhibited PDE (PDE3) inhibitor, siguazodan, had little or no effect (IC_{40} > 100 μM) on anti-CD3-stimulated release of either IL-4 or IL-5 and did not significantly enhance the inhibitory action of RP 73401 on the release of either of these cytokines. Finally, RP 73401 (IC_{50}: 0.41 ± 0.19 nM) inhibited anti-CD3-stimulated DNA synthesis in splenocyte preparations from M. *corti*-infected mice and siguazodan (10 μM) had no effect on this response, either alone or in combination with the PDE4 inhibitor. The results show that PDE4 inhibitors suppress the release of Th2 cytokines from anti-CD3-stimulated murine spenocytes and that this effect is correlated with inhibition of a low-affinity PDE4 form. BIOCHEM PHARMACOL **58**;6:991–999, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cAMP phosphodiesterase; RP 73401; rolipram; interleukin-4; interleukin-5; M. *corti*; murine splenocytes; anti-CD3; DNA synthesis

Inhibitors of PDE4‡ have attracted much interest during the past few years as potential anti-asthma drugs [1, 2]. The interest in the anti-asthma potential of PDE4 inhibitors stems from their wide-ranging suppressive actions on the functions of inflammatory cells implicated in this disease, such as eosinophils [3, 4], mononuclear phagocytes [5], mast cells [6], and basophils [7], as well as endothelial [8, 9] and epithelial cells [10, 11]. T lymphocyte responses have also been reported to be suppressed by PDE4 inhibitors. In particular, proliferation and IL-2 release induced by a variety of stimuli are reduced, although in some [e.g. 12], but not in all [13], cases, total ablation of these Th1 responses is only achieved in combination with a cGMP-inhibited phosphodiesterase (PDE3) inhibitor.

The effects of PDE4 inhibitors on Th2 cytokines, IL-4, and IL-5, which orchestrate the humoral immunoglobulin E and cellular (eosinophilic) responses to exogenous antigen, respectively [14], are less clear. Some studies [15–19] suggest that agents that elevate cAMP are only slightly effective in suppressing the release of Th2 cytokines from human peripheral blood T cells and splenocytes. There are even reports that cAMP up-regulates IL-4 and IL-5 production from activated, spleen-derived, murine $CD4^+$ T cells as well as cultured T cell lines, while decreasing IL-2 release [20, 21]. However, recent reports indicate that T cell proliferative responses to Th2 antigenic stimuli, as well as the release of IL-4 and IL-5, can be suppressed by PDE4

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‡ *Abbreviations:* PDE, phosphodiesterase; cAMP, adenosine 3′,5′-cyclic monophosphate; HARBS, high-affinity rolipram binding site, IL, interleukin; LA-PDE4, low-affinity PDE4; HA-PDE4, high-affinity PDE; PGE_2, prostaglandin E_2; IFN, interferon; PBMC, peripheral blood mononuclear cells; Th cells, T helper cells; FBS, foetal bovine serum; CD3, cluster of differentiation antigen 3; and Staph. A, Staphylococcal enterotoxin A.

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inhibitors [22–27]. The reasons for these discrepancies are uncertain, but may relate to differences in the stimulus and the origins of the T cells employed in the studies.

In the present paper, the effects of PDE4 inhibitors on anti-CD3-stimulated IL-4 and IL-5 release from anti-CD3-treated splenocytes of *Mesocestoides* (M.) *corti*-infected BALB/c mice have been investigated. Infection of mice with parasitic helminth worms elicits a Th2 response that is characterised by eosinophilia and elevated serum antibody concentrations [28, 29]. Splenocytes isolated from infected animals release abundant quantities of IL-4 and IL-5 when activated with anti-CD3, which cross-links the T cell receptor (TCR), making this a good model system in which to test the effects of PDE4 inhibitors on Th2 responses. The results show that PDE4 inhibitors, such as RP 73401 (piclamilast), suppress IL-4 and IL-5 release, although compete inhibition is not achieved, particularly for IL-5. This effect is better correlated with inhibition of a form of PDE4 with which rolipram interacts with low-affinity (LA-PDE4) than with a 'high-affinity' form as represented by displacement of [^{3}H] *R*(−)-rolipram from its high-affinity binding site (HARBS/HA-PDE4) [30]. Furthermore, PDE3 inhibitors exert little influence on the suppressive effects of RP 73401 on the anti-CD3-induced IL-4/IL-5 elaboration and, interestingly, the proliferative response to the same stimulus.

MATERIALS AND METHODS

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 synthesised by the Department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd. Ibudilast (3-isobutyryl-2-isopropylpyrazolo [1,5-α]pyridine, KC-404) was a gift from Kyorin Pharmaceutical Co. Ltd. Ro 20-1724 (1-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) was obtained from Roche Products Ltd. 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. Trequinsin (9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-α]isoquinolin-4-one, HL-725) was purchased from Calbiochem-Novabiochem U. K. Ltd. The enantiomers of (±) rolipram were separated as described by Schneider *et al.* [31]. Rat anti-mouse IL-2 neutralising monoclonal antibody, rat anti-mouse IL-4 monoclonal capture antibody, biotinylated rat anti-mouse IL-4 monoclonal antibody, and recombinant murine IL-4 (20,000 U/mL) were from Pharmingen (Cambridge Bioscience). Recombinant mouse IL-2 was from Genzyme Diagnostics. Rat anti-mouse IL-5 capture antibody (TRFK-5), biotinylated rat anti-mouse IL-5 antibody (TRFK-4), and recombinant murine IL-5 (16,000 U/mL) were purchased from British Biotechnology. [Methyl-^{3}H] rolipram (50–86 Ci/mmol), [methyl, 1′,2′-^{3}H] thymidine 5′-triphosphate ([^{3}H] dTTP (90–130 Ci/mmol), and cyclic [2,8-^{3}H] AMP (41 Ci/mmol) were from Amersham International. Cell culture reagents were from GIBCO BRL, tissue culture plates (Nunc) from Life Technologies Ltd., immunoplates from Costar, and 24- and 96-well filtration (0.22 μm) plates from Millipore, U.K. Ltd. All other chemicals were obtained from Sigma Chemical Co., BDH Chemicals, and Rhône-Poulenc Ltd.

Treatment of Mice with M. corti

Packed worms (10 mL) were stored in PBS (total volume, 50 mL). The PBS was replaced each week and the worms placed in clean flasks. The viability of the worms was checked under an inverted microscope. Worms were transfered to a Universal tube and allowed to settle before PBS was aspirated off. The packed worms were taken up into a 1-mL syringe and 50 μL was injected (i.p.) into female BALB/c mice (~20 g). The mice were left for 19–23 days before being killed for their spleens.

Preparation of Splenocytes

Spleens from infected mice were removed from naive BALB/c mice, placed in RPMI 1640 medium with Glutamax 1 and HEPES (25 mM) (GIBCO BRL), containing (FBS, 10%), penicillin/streptomycin (100 U/mL) and β-mercaptoethanol (50 μM) and pressed through a 70-micron cell strainer (Falcon, U. K.) into a plastic tube (50 mL). The splenocytes were centrifuged (1000 *g*, 10 min) and resuspended in erythrocyte lysing buffer (NH_4Cl, 0.154 M; $KHCO_3$, 0.012 M; EDTA, 0.1 mM). After 10 min, the cells were centrifuged (1000 *g*, 10 min), resuspended, and washed twice in RPMI medium and cultured overnight (37°, 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

Flat-bottomed, 96-well microtitre plates (Nunc) were coated with anti-CD3 antibody (50 μL/well, 5 μg/mL) in coating buffer and incubated for 24 hr at 4°. The plates were then washed twice with RPMI medium containing FBS (10%). Splenocytes were resuspended in RPMI medium at a concentration of 2×10^6 cells/mL. A portion (160 μL) of this cell suspension was added to each anti-CD3 precoated well and incubated with compounds or vehicle (dimethyl sulphoxide, 0.1%; total volume 200 μL)

for 48 hr at 37° in a humidified atmosphere of CO_2. At the end of this incubation period, the plates were centrifuged (500 *g*, 10 min) and 150 μL of the supernatant was removed into the corresponding wells of another microtitre plate and stored frozen (−20°) prior to determination of IL-4 and IL-5.

Measurement of IL-4 and IL-5

For measurement of IL-4, 96-well microtitre plates (Costar enzyme immunoassay/radioimmunoassay) were coated with 50 μL/well of purified rat anti-mouse IL-4 monoclonal antibody (capture antibody, 2 μg/mL) in coating buffer for 48 hr at 4°. The plates were washed 4 times with 150 μL of PBS containing Tween 20 (0.05%, v/v), and additional sites were blocked with 150 μL of coating buffer containing BSA (1%) for 30 min. The plates were again washed 4 times with PBS/Tween 20 solution, and 100 μL of recombinant, murine IL-4 standard (12.5–0.195 U/mL) or an aliquot of splenocyte supernatant was added per well and incubated at 37° for 2 hr. Plates were washed as above and 100 μL/well of biotinylated rat anti-mouse IL-4 monoclonal antibody (Pharmingen, 0.25 μg/mL) in PBS/Tween 20 containing BSA (1%) was added and incubated at 37° for 1.5 hr. After washing the plates as above, 100 μL/well of streptavidin horseradish peroxidase enzyme (AMS Biotechnology, 0.4 μg/mL) was added and incubated at 37° for 30 min. The plates were washed and 100 μL/well of substrate (3,3′,5′,5-tetramethyl benzidine tablet and 0.0006% H_2O_2 in phosphate/citrate buffer) was added and incubated at room temperature for 7–12 min. Colour development was stopped with 100 μL H_2SO_4 (2M), and absorbance at 450 nm was measured on a Titertek Multiscan ICN MCC/340 plate reader. IC_{40} values (concentration which produced 40% inhibition of IL-4 release) are quoted, since complete suppression of the response was not achieved with PDE4 inhibitors and the 40% inhibition lay on the linear portion of the concentration–response curves. IL-5 was measured similarly. The rat anti-mouse IL-5 capture antibody (TRFK-5) and the biotinylated, rat anti-mouse IL-5 antibody (TRFK-4) were diluted 1:500 in coating buffer prior to use.

Measurement of [^{3}H] Thymidine Incorporation into Splenocytes

Splenocytes prepared from *M. corti*-infected mice as above were dispensed into anti-CD3-coated, 96-well microtitre plates (3.2×10^5 cells/160 μL) containing compounds (20 μL in culture medium) as in the IL-4/IL-5 release experiments and cultured for 24 hr. [^{3}H] Methylthymidine (1 μCi, 20 μL) was then added to each well and, after 18 hr, the cells were collected on membrane filters using a Packard Filtermate 196 Cell Harvester. [^{3}H] Thymidine incorporation into the cells was measured using 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°, 5% CO_2) in RPMI–Glutamax I medium (GIBCO) supplemented with 5% FBS, penicillin (50 U/mL)/streptomycin (50 μg/mL), β-mercaptoethanol (50 μM), and IL-2 (10 ng/mL). Cells (10^8) were centrifuged (250 *g*, 10 min) and washed twice with 50 mL PBS. The cell pellet was resuspended in ice-cold homogenisation buffer (Tris-HCl 20 mM [pH 7.5]; $MgCl_2$, 2mM; dithiothreitol, 1 mM; EDTA, 5 mM; sucrose, Triton X-100, 0.1%; sucrose, 0.25 M; *p*-tosyl-L-lycine chloromethyl ketone [TLCK], 10 μM; leupeptin, 10 μg/mL; aprotinin, 2000 U/mL) and homogenised using a Dounce homogeniser (20 strokes). The homogenate was used in PDE assays.

Measurement of PDE Activity

PDE activity was determined by the two-step radioisotope method of Thompson *et al.* [32]. The reaction mixture contained: Tris–HCl, 20 mM (pH 8.0); $MgCl_2$, 10 mM; 2-mercaptoethanol, 4 mM; EGTA, 0.2 mM; BSA, 0.05 mg/mL, and [^{3}H] cAMP (1 μM, 100,000 cpm/assay). 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 (concentrations which produced 50% inhibition of substrate hydrolysis) for the compounds were determined from concentration (0.1 nM to 40 μM)–response curves.

Categorisation of PDE Isoenzymes

The nomenclature adopted in this paper for the different cyclic nucleotide PDEs is based on that of Beavo and Reifsnyder [33].

Measurement of [^{3}H] (±) Rolipram Binding to Soluble Fraction of Mouse Brain

Brains from BALB/c mice were homogenised in homogenisation buffer (Tris–HCl, 50 mM, pH 7.4; $MgCl_2$, 5 mM; dithiothreitol, 0.1 mM; TLCK, 20 μM) and a soluble fraction prepared (30,000 *g*, 30 min; high-speed 65M Europa Ultracentrifuge). The binding assay was performed on 24-well plate GF/B Unifilter plates soaked in 0.3% polyethylenimine (PEI) (cytosolic assay) using a Packard Harvester Filtermate 196 fitted with a Unifilter-24 probed head (Canberra Packard), essentially as described previously by Schneider *et al.* [31] with [^{3}H] *R*-(−)-rolipram (2 nM) and cytosolic samples corresponding to 500 μg of original brain tissue.

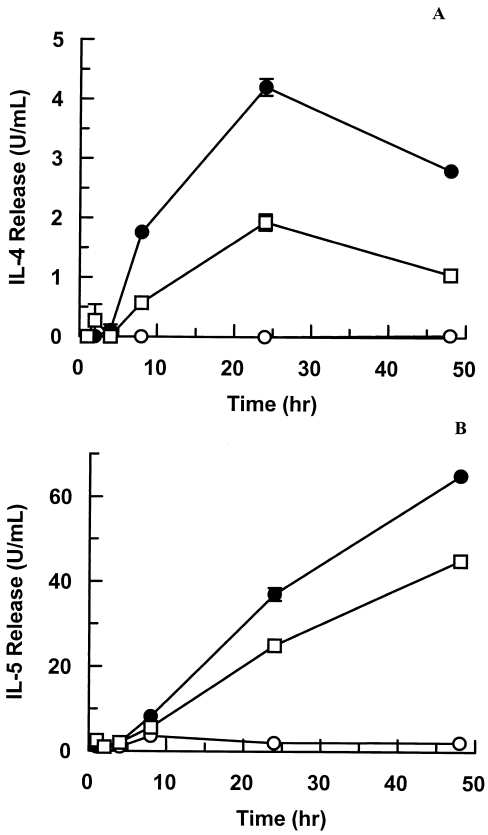

FIG. 1. Time–course for suppression of anti-CD3-stimulated IL-4 (panel A) and IL-5 (panel B) release from splenocytes; (○) no stimulation, (●) anti-CD3 alone, and (□) anti-CD3 + 0.1 μM RP 73401. The results represent the means ± SEM of a typical experiment performed in quadruplicate.

Statistical Analysis

Data are presented as means ± SEM 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-4 release by several standard and Rhône-Poulenc Rorer compounds and inhibition of PDE4 or displacement of [^{3}H] rolipram from brain membranes, determination of linear (r^2) correlations of the respective $\log_{10}$M values were determined using the Prism program (GraphPad Software Inc.).

RESULTS

Inhibition of IL-4 and IL-5 Generation

Anti-CD3 was a powerful stimulus for the elaboration of both IL-4 and IL-5 from splenocytes of *M. corti*-infected mice with 4–10 U/mL and 40–80 U/mL, respectively, of the two cytokines being routinely detected in media at the end of experiments in the absence of other additions (Fig. 1, A and B). Anti-CD3-treated splenocytes from *M. corti*-infected mice consistently elaborated higher and more reproducible levels of IL-4 and IL-5 than cells from non-infected mice. Levels of IL-4 in the supernatant reached peak levels more quickly than those of IL-5 (Fig. 1). IL-2 levels were either very low or undetectable in supernatants from these cell incubations. RP 73401 (IC_{40}: 0.011 ± 0.004 μM, N = 9) was a very potent inhibitor of anti-CD3-induced IL-4 release, being ~40-fold more potent than (±)-rolipram (IC_{40}: 0.43 ± 0.091 μM, N = 3) (Fig. 2A; Table 1). Maximal suppression of IL-4 release by PDE4 inhibitors was consistently 60–70%. In a time–course study (Fig. 1), RP 73401 (0.1 μM) achieved similar levels of inhibition when incubations were terminated at 8, 24, or 48 hr; however, the PDE4 inhibitor was progressively less effective when added at increasing time periods after exposure of the cells to anti-CD3 (data not shown). *R*-(−)-rolipram (IC_{40}: 0.39 ± 0.091 μM, N = 3) was ~6-fold more potent than *S*-(+)-rolipram (IC_{40}: 2.6 ± 0.95 μM, $N = 3$) in inhibiting the anti-CD3-induced response (Fig. 2B). RP 73401 and rolipram also inhibited anti-CD3-induced IL-5 release over the same concentration range as for inhibition of IL-4 (Fig. 2, C and D), although the maximal inhibition achieved was consistently only about ~40%.

The potency difference (40-fold) between RP 73401 and (±)-rolipram in suppressing anti-CD3-induced IL-4 release was not as great as that observed for inhibition of CTLL cell PDE4 (a convenient source of LA-PDE4) (264-fold), but contrasts markedly with that for displacement of [^{3}H] *R*-(−)-rolipram from HARBS, against which the two compounds were equipotent (Table 1). Trequinsin showed similar activity to rolipram in inhibiting PDE4 catalytic activity and IL-4 release, but was over 300-fold less potent in competing with rolipram for HARBS (Table 1). The rolipram enantiomeric potency difference (6-fold) for IL-4 suppression was intermediate between that observed for CTLL cell PDE4 inhibition (4.3-fold difference) and displacement of [^{3}H] *R*-(−)-rolipram from the mouse brain-soluble fraction (8-fold difference) (Table 1, Fig. 2B).

Studies were performed to determine whether inhibition of IL-4 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 soluble fraction of mouse brain. A closer correlation ($r^2 = 0.82$, $P < 0.01$, $N = 13$) existed between suppression of IL-4 release by PDE inhibitors and inhibition of CTLL cell PDE4 (Table 1, Fig. 3A) than between suppression of IL-4 release and displacement of [^{3}H] *R*-(−)-rolipram from its binding site in the brain soluble fraction ($r^2 = 0.26$, $P < 0.01$, N = 12) (Table 1, Fig. 3B).

Zaprinast, a selective PDE5 inhibitor, had little effect on anti-CD3-induced IL-4 (7.0 ± 9.0% inhibition at 100 μM, N = 3) or IL-5 release (15 ± 5.0% inhibition at 100 μM, N = 3). The PDE3 inhibitor siguazodan by itself also had little effect on anti-CD3-induced IL-4 (19 + 6.0% inhibition at 100 μM, N = 3) or IL-5 release (14 ± 3.0% inhibition at 100 μM, N = 3), and a submaximal concentration of siguazodan (10 μM) failed to significantly potentiate the suppressive effects of RP 73401 on the release of either Th2 cytokine (data not shown). PGE_2, which stim-

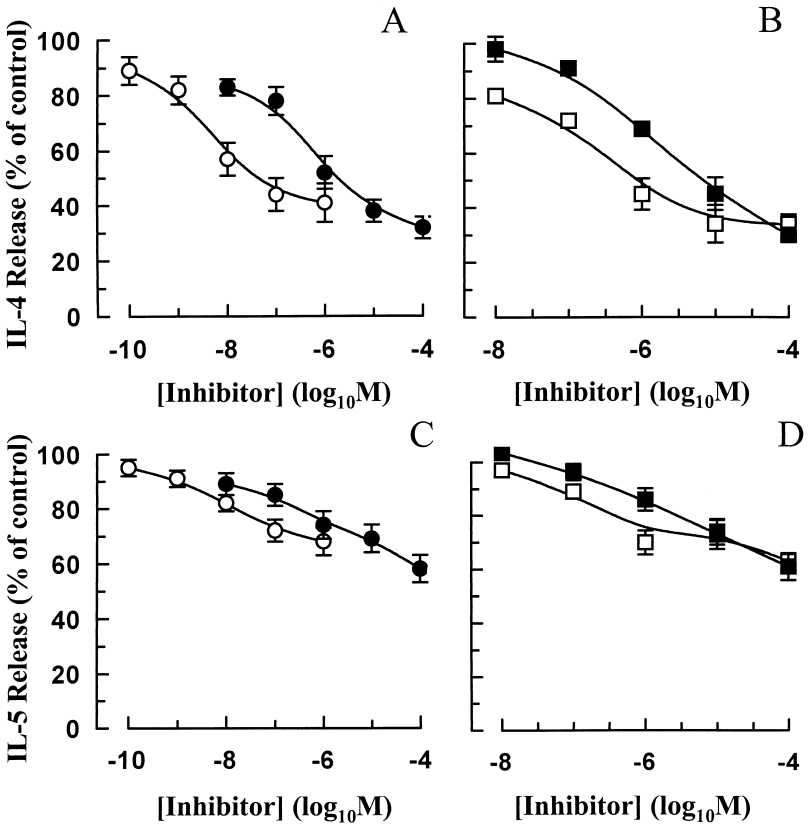

FIG. 2. Inhibition of anti-CD3-induced IL-4 (panels A and B) and IL-5 release (panels C and D) by RP 73401 (○) and (±)-rolipram (●) (panels A and C) and by *R*-(−)-rolipram (□) and *S*-(+) rolipram (■) (panels B and D). The results represent the means ± SEM of 3–4 separate experiments performed in quadruplicate in different preparations of cells.

ulates cAMP accumulation through activation of adenylyl cyclase, suppressed IL-4 (IC_{40}: 0.5 μM, maximal inhibition of 52% at 1 μM) but had little effect on IL-5 release (16% inhibition at 100 μM) (data not shown). A threshold concentration (10 nM) of PGE_2 failed to potentiate the action of RP 73401 on either Th2 cytokine. Dexamethasone (0.1 μM) or cyclosporine (0.1 μM) inhibited anti-CD3-induced IL-4 and IL-5 generation by greater than 95%.

RP 73401 has previously been shown to be a very potent inhibitor of Staph. A-induced IL-2 release by splenocytes from naive mice [13]. Although little or no IL-2 was detectable in supernatants from anti-CD3-stimulated splenocytes from *M. corti*-infected mice, experiments were

TABLE 1. Potencies of PDE4 inhibitors in inhibiting CTLL cell PDE4, displacing [^{3}H] rolipram binding, and suppressing splenocyte IL-4 release

Compound	PDE4 inhibition (IC_{50}-μM)	Displacement of [^{3}H] rolipram (Ki_{app}-μM)	Suppression of IL-4 release (IC_{40}-μM)
RP 73401	0.0011 ± 0.00015	0.0010 ± 0.00010	0.011 ± 0.00020
R-(−)rolipram	0.30 ± 0.045	0.00070 ± 0.00010	0.39 ± 0.091
S-(+)rolipram	1.3 ± 0.55	0.0055 ± 0.0013	2.6 ± 0.95
(±)-Rolipram	0.29 ± 0.11	0.0015 ± 0.00010	0.43 ± 0.091
Denbufylline	0.41 ± 0.19	0.0032 ± 0.00020	2.5 ± 1.5
Ro 20-1724	2.8 ± 1.9	0.016 ± 0.0029	2.5 ± 0.30
Ibudilast	0.85 ± 0.25	0.0046 ± 0.00010	0.59 ± 0.050
IBMX	8.0 ± 2.0	0.54 ± 0.013	11 ± 0.50
Trequinsin	0.71 ± 0.21	2.7 ± 0.36	1.1 ± 0.10
Compound A	0.0071 ± 0.0022	ND	0.0078
Compound B	0.0034 ± 0.0015	0.0078 ± 0.0029	0.080
Compound C	0.0014 ± 0.00030	0.0031 ± 0.00060	0.030
Compound D	0.014 ± 0.0018	0.015 ± 0.0020	0.20

cAMP 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 soluble fraction of mouse brain. IL-4 release from murine splenocytes was stimulated with anti-CD3 antibody as described in the Methods section. The results represent the means ± SEM of 2–4 experiments. ND, not determined.

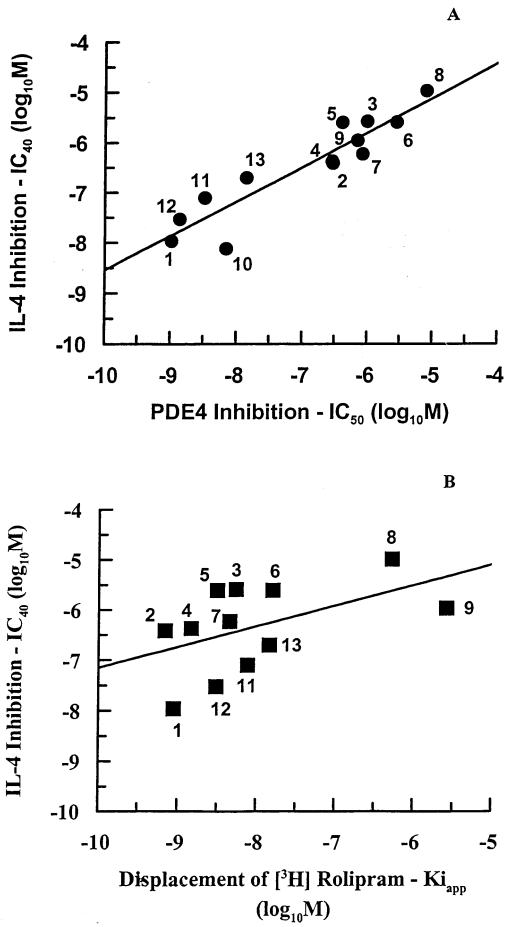

FIG. 3. Inhibition of anti-CD3-induced IL-4 release as function of inhibition of CTLL cell PDE4 (panel A) and displacement of [^{3}H] *R*-(−)-rolipram binding to the soluble fraction of murine brain (panel B). The results are taken from Table 1. Rolipram binding data are expressed as a Ki_{app} value ($log_{10}M$), PDE4 data as IC_{50} values ($log_{10}M$), and IL-4 data as IC_{40} 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.

conducted to eliminate the slim possibility that further reduction of the very low release of this T cell mitogen might account for the suppression of Th2 cytokines by RP 73401. The failure of either an anti-IL-2 neutralising antibody (7 μg/mL) or of exogenously added IL-2 (20 U/mL) to significantly affect the inhibition achieved by RP 73401 (data not shown) confirmed that inhibition of IL-4 release by the PDE4 inhibitor was not via ablation of IL-2 release.

Inhibition of DNA Synthesis

Because previous studies demonstrated that PDE4 inhibitors synergise with PDE3 inhibitors in inhibiting mitogen-induced T lymphocyte proliferation [12, 13], we investigated the effects of RP 73401, alone and in combination with siguazodan (10 μM), on anti-CD3-induced incorporation of [^{3}H] thymidine into the DNA of murine splenocytes. As shown in Fig. 4, RP 73401 (IC_{50}: 0.41 ± 0.19 nM) potently suppressed DNA synthesis with a maximal 80% inhibition being observed at 0.1 μM. Siguazodan (10 μM) had no significant effect on [^{3}H] thymidine incorporation either alone or in combination with RP 73401 (Fig. 4). Dexamethasone (0.1 μM) and cyclosporin A (0.1 μM) inhibited anti-CD3-induced DNA synthesis by greater than 90%.

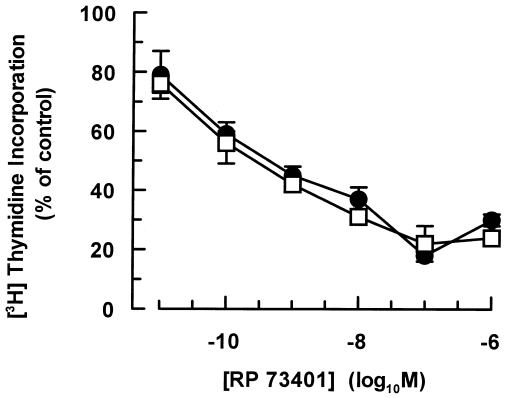

FIG. 4. Lack of potentiation by siguazodan of RP 73401 inhibition of anti-CD3-induced [^{3}H] thymidine incorporation into murine splenocytes. Cells were stimulated with anti-CD3 in the presence of RP 73401 alone (□) or RP 73401 plus siguazodan (10 μM) (●) as described in the Methods section. The results represent the means ± SEM of a typical experiment performed in quadruplicate.

DISCUSSION

The studies reported in this paper demonstrate that PDE4 inhibitors potently suppress anti-CD3-induced release of IL-4 and, to a lesser degree, IL-5 from splenocytes of *M. corti*-infected mice. The incomplete suppression of the response by PDE4 inhibitors differed from the actions of glucocorticoids and the immunosuppressant, cyclosporine, which virtually completely inhibited Th2 cytokine release. These results indicate that as well as the well-known effects of PDE4 inhibitors in suppressing the Th1 cytokine, IL-2, the generation of Th2 cytokines, particularly IL-4, is also attenuated. Whilst PDE4 inhibitors, such as RP 73401, are potent inhibitors of IL-2 release from splenocytes [13], our current data indicate that their effects on Th2 cytokine release are not mediated indirectly by inhibiting IL-2. Support for the current findings derives from previous studies in freshly isolated, $CD8^+$-depleted murine splenocytes, in which concanavalin A (Con A)-induced IL-4 release is potently inhibited by PGE_2, an activator of adenylate cyclase [26]. However, studies with other lymphocyte preparations have failed to paint a coherent picture regarding Th2 cytokine responses to PDE4 inhibitors. Several reports indicate that agents that elevate or mimic cAMP are far more effective in suppressing the release of Th1 cytokines (IL-2, IFNγ) than Th2 cytokines (IL-4,

IL-5) [15–17, 19]. For example, PDE4 inhibitors and dibutyryl cAMP were reported to potently suppress the release of IL-2 and IFNγ from mitogen (phytohemagglutinin, PHA)-stimulated human PBMCs, but elicit only weak effects on IL-4 and IL-5 [24]. Similarly, the weak and non-selective PDE inhibitor, pentoxifylline, reduces antigen (major basic protein)- and mitogen (Con A)-induced IL-2 release from rat T cell lines established from lymph node cells, but is without effect on mitogen-induced IL-4 elaboration [16]. In murine lymph node-derived T cells and certain Th2 cell lines, agents that increase or mimic cAMP actually increase IL-4 and/or IL-5 production. For example, in anti-CD3-treated D10,G4.1 (D10) murine Th2 cells, rolipram, alone or in combination with the PDE3 inhibitor motapizone, increases production of IL-5 but not IL-4 [34]. In the mouse thymoma cell line EL-4, dibutyryl cAMP augments the IL-5 mRNA expression and protein production elicited by phorbol 12-myristate 13-acetate (PMA), but completely inhibits the phorbol ester-induced production of IL-2 and, to a lesser extent, IL-3, IL-4, and IL-10 [21]. To add further complexity, agents that elevate cAMP, including the PDE4 inhibitor Ro 20-1724, increase IL-4 as well as IL-5 production while inhibiting IL-2 release from lymph node-derived $CD4^+$ T cells stimulated with PMA and the calcium ionophore, ionomycin [20]. These effects on protein generation correlate closely with mRNA expression [20].

In contrast to these studies, several reports indicate that PDE4 inhibitors can suppress Th2 cytokines. For example, rolipram is more effective in blocking the proliferation of PBMCs elicited by ragweed (Th2) antigen than tetanus toxoid (Th1) antigen [35], and reverse transcriptase-polymerase chain reaction showed attenuation of IL-5 and IFNγ, but not IL-4, gene expression following allergen provocation [36]. Clonal proliferative responses in Th2 cells derived from atopic asthmatics are more sensitive to the inhibitory effects of rolipram than those of Th1 cells, and ragweed-induced IL-4, IL-5, and IFNγ production is suppressed by the PDE4 inhibitor [35]. In human whole blood stimulated with PHA, rolipram and nitraquazone (PDE4 inhibitor) are 3- to 6-fold more potent in reducing production of IL-5 than IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFNγ [27]. Furthermore, WAY PDA-641 (PDE4 inhibitor) suppresses anti-CD3-stimulated IL-4 release from a T cell line derived from atopic donors [37]. Further clouding the issue are studies with the novel PDE4 inhibitor, T-440, in which suppression of IL-5, IL-4, and IL-2 release from PBMCs was observed [22, 23].

The effects of PDE4 inhibitors on Th2 cytokine production may be different in purified T cell populations compared to mixed cell populations such as the splenocyte preparations under investigation in the present studies, although this in itself is insufficient to explain the plethora of apparently discrepant results detailed above. As well as T helper cells, the splenocyte population includes $CD8^+$ T cells, B cells, and monocytes, and indirect actions via these may be responsible for at least some of the effects of PDE4 inhibitors on IL-4 and IL-5 release. Although this has not been addressed in the present studies, a precedent for such an indirect action of PDE4 inhibitors is the recent demonstration that the inhibition of IL-4-stimulated immunoglobulin (Ig) E in human PBMCs from non-allergic donors by rolipram and Ro 20-1724 is not direct on B lymphocytes, but is dependent on the presence of monocytes [38]. However, in contrast to earlier studies, it is now clear that direct, suppressive effects of PDE4 inhibitors on Th2 cytokine release can occur. Besides the possible influence that other immunocompetent cells may or may not exert, the actions of PDE4 inhibitors may be affected by a number of factors determined by the history of the cells under study and the exogenous stimuli that can alter the PDE profile of the T cells and/or the responsiveness to cAMP of downstream elements controlling gene expression of Th2 cytokines.

Several functional effects of PDE4 inhibitors, including those which have hindered their development as potentially important anti-inflammatory agents, are closely associated with their affinities for a PDE4 form for which rolipram exhibits low nM affinity (HA-PDE4), as represented by HARBS [30]. However, it is becoming clear that some potentially beneficial functional effects of PDE inhibitors are more closely correlated to the potencies of these compounds in inhibiting a form of PDE4 which is relatively insensitive to the actions of rolipram ($IC_{50} > 100$ nM) and therefore designated low-affinity PDE4 (LA-PDE4) [30]. For example, suppression of tumor necrosis factor-α release from human monocytes [5, 39] and inhibition of IL-2 release from Staph. A-stimulated murine splenocytes [13] are better correlated with inhibition of LA-PDE4 catalytic activity than competition for HARBS. We have now extended this further in demonstrating that another effect of PDE4 inhibitors on T cells, namely suppression of IL-4 release from anti-CD3-stimulated splenocytes, is also closely associated with inhibition of LA-PDE4. The lack of the functional importance of HARBS in modulating IL-4 release is exemplified by the greater than 40-fold greater potency of RP 73401, a compound that is proposed not to discriminate between different PDE4 conformers [30], compared to the HA-PDE4-selective rolipram. Both compounds exhibit similar affinities for HARBS, whereas RP 73401 is much more potent in inhibiting LA-PDE4 (Table 1, [30]). Furthermore, trequinsin, which interacts weakly with HARBS compared to rolipram (100-fold less potent), is equipotent with the archetypal PDE4 inhibitor in suppressing splenocyte IL-4 release, which mirrors the relative potencies of the two compounds against LA-PDE4. It should be stressed that the potency difference between RP 73401 and rolipram in inhibiting IL-4 release is smaller than that for suppressing IL-2 release ([13], data reported herein). Furthermore, the correlation between inhibition of LA-PDE4 and suppression of IL-2 release [13] is stronger than that for inhibition of LA-PDE4 and suppression of

IL-4 release, suggesting, perhaps, that different PDE4 forms may be responsible for regulating Th1 and Th2 responses.

As in the case of Staph. A-induced IL-2 release [13], siguazodan failed to influence the potent inhibitory effect of RP 73401 on anti-CD3-elicited IL-4 and IL-5 release and did not alter the maximum inhibition achieved. Siguazodan also failed to influence the inhibition by RP 73401 of anti-CD3-induced [^{3}H]thymidine incorporation into splenocytes. This contrasts with results on Staph. A-induced DNA synthesis in murine splenocytes, which was relatively poorly suppressed by RP 73401 alone and whose action was clearly potentiated by the PDE3 inhibitor [13]. Although dual actions of PDE4 and PDE3 inhibitors on T cell proliferation have been reported previously in a mixed population of purified, human T lymphocytes [40], highly purified preparations of human $CD4^+$ and $CD8^+$ T cells [12], and in murine splenocytes [41], inhibition of ragweed or tetanus toxoid-induced proliferation of PBMCs from atopic individuals by rolipram is not enhanced by PDE3 inhibitors [35]. Once again, the history of the T cell donor as well as the tissue source (peripheral blood, thymus, spleen, lymph nodes, etc.) is likely to influence the properties of the T cell population(s) that are clonally expanded by the antigenic/mitogenic stimulus. It is likely that the different clones will have distinct PDE profiles which may influence the antiproliferative efficacy of isozyme-selective inhibitors. Furthermore, diverse stimuli may preferentially induce proliferation of different populations of T cells with distinct PDE profiles and, indeed, differentially change the PDE complement, as has been demonstrated in Jurkat T cells [42]. Thus, whether antigen/mitogen-induced T cell proliferation, as well as cytokine release, can be suppressed by PDE4 inhibitors alone or only in combination with a PDE3 inhibitor will be influenced by a plethora of factors.

In summary, the results demonstrate that anti-CD3-induced IL-4 release from splenocytes of *M. corti*-infected mice is inhibited by PDE4 inhibitors, albeit incompletely, and that this effect is better correlated with inhibition of LA-PDE4 than affinity for HA-PDE4. IL-5 release is only weakly inhibited by PDE4 inhibitors. The inability of siguazodan to influence the inhibitory effects of RP 73401 on IL-4 and IL-5 generation as well as on [^{3}H] thymidine incorporation into DNA of the splenocytes indicates a lack of functional importance of PDE3 in the anti-CD3 responsive T cell population. Whether suppressive actions on Th2 cytokines contribute to the anti-inflammatory actions of PDE4 inhibitors in antigen-driven animal models of asthma remains to be demonstrated.

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