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# Therapeutic Potential of Phosphodiesterase-4 and -3 Inhibitors in Th1-Mediated Autoimmune Diseases

Bibiana Bielekova, Anne Lincoln, Henry McFarland, and Roland Martin<sup>1</sup>

Phosphodiesterase-4 (PDE4) inhibitors have the potential to modulate immune responses from the Th1 toward the Th2 phenotype and are considered candidate therapies for Th1-mediated autoimmune disorders. However, depending on the model and cell types employed, studies of atopic individuals have come to the opposite conclusion, i.e., that PDE inhibitors may be beneficial in asthma. Using *in vitro* immunopharmacologic techniques we analyzed the effects of PDE4 and PDE3 inhibitors on human immune cells to address these discrepancies and broaden our understanding of their mechanism of action. Our results indicate that PDE inhibitors have complex inhibitory effects within *in vivo* achievable concentration ranges on Th1-mediated immunity, whereas Th2-mediated responses are mostly unaffected or enhanced. The Th2 skewing of the developing immune response is explained by the effects of PDE inhibitors on several factors contributing to T cell priming: the cytokine milieu; the type of costimulatory signal, i.e., up-regulation of CD86 and down-regulation of CD80; and the Ag avidity. The combination of PDE4 and PDE3 inhibitors expresses synergistic effects and may broaden the therapeutic window. Finally, we observed a differential sensitivity to PDE inhibition in autoreactive vs foreign Ag-specific T cells and cells derived from multiple sclerosis patients vs those derived from healthy donors. This suggests that PDE inhibition weakens the strength of the T cell stimulus and corrects the underlying disease-associated cytokine skew in T cell-mediated autoimmune disorders. These new findings broaden the understanding of the immunomodulatory actions of PDE inhibitors and underscore their promising drug profile for the treatment of autoimmune disorders. *The Journal of Immunology*, 2000, 164: 1117–1124.

Hyperactive Th1-mediated immune responses are thought to be involved in the pathogenesis of many autoimmune diseases, including multiple sclerosis (MS).<sup>2</sup> Phosphodiesterases (PDE) are enzymes degrading the second messenger cAMP, which mediates and regulates essential intracellular processes (1). There are 10 different PDE families, but immune cells predominantly express families PDE4, PDE3, and, to a lesser extent, PDE7 (2, 3). Although no PDE7 inhibitor is available, the inhibitors of PDE4 and PDE3 families exert complex immunomodulatory properties. In animals, these drugs inhibit Ag-mediated T cell proliferation and skew the T cell cytokine profile toward a Th2 phenotype by down-regulating the expression or production of Th1 cytokines (4–6) and have no effect or even augment the production of Th2 cytokines (6, 7). These properties render PDE inhibition a candidate therapy for Th1-mediated autoimmune disorders. Indeed, both nonselective as well as PDE4-specific inhibitors were effective in ameliorating disease in different experimental autoimmune encephalomyelitis models (5, 8–10) and in collagen-induced arthritis models (11, 12). However, the simple extrapolation of therapeutic efficacy from animal models to human disorders is not easily feasible (13), and therefore, the analysis of the immunomodulatory properties of PDE inhibitors on human immune cells is an important step in preclinical testing.

Studies exploring the effects of PDE inhibitors in humans *in vitro* or *in vivo* are still limited. Although there is some evidence for a preferential inhibition of proinflammatory cytokines in Th1-mediated human autoimmune conditions (2, 14, 15), data from asthmatic and atopic individuals reached almost the opposite conclusion, i.e., that these drugs lead to preferential inhibition of Th2 responses (16). We therefore decided to study the effects of the selective PDE4 inhibitor rolipram and the PDE3 inhibitor cilostamide on a large number of human autoreactive and foreign Ag-reactive T cell lines (TCL) derived from MS patients and healthy individuals and asked the following questions. 1) Which biological functions (Ag-specific proliferation, cytokine production, functional Ag avidity) of human CD4<sup>+</sup> T cells are influenced by these PDE inhibitors? 2) Can these effects be induced by the concentrations of drugs achievable *in vivo*? 3) Is there a differential effect between PDE4 and PDE3 inhibitors and their combination? 4) Is there a differential sensitivity to the PDE inhibition between autoreactive and foreign Ag-reactive TCL and between TCL derived from MS patients vs healthy donors? Finally, 5) what are the possible explanations of the immunomodulatory effect of PDE inhibitors?

## Materials and Methods

### *Reagents, generation of T cell lines, and proliferation assays*

Rolipram (racemate of 4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone) was provided by Dr. Harald Dinter (Berlex Laboratories, Richmond, CA). Cilostamide (OPC 3689) was a gift from Dr. Vincent Manganiello Pulmonary-Critical Care Medicine Branch (PCCMB), National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). Fresh solutions of individual drugs were prepared for each experiment. The solvent for both drugs, DMSO (Sigma, St. Louis, MO), was used in 1/1000 dilution with T cell medium for the 10- $\mu$ M concentration of drugs and at this concentration did not influence T cell proliferation when used as a negative control.

Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

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<sup>1</sup> Address correspondence and reprint requests to Dr. Roland Martin, Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 10, Room 5B-16, 10 Center Drive MSC 1400, Bethesda, MD 20892-1400. E-mail address: rmartnib@box-r.nih.gov

<sup>2</sup> Abbreviations used in this paper: MS, multiple sclerosis; PDE, phosphodiesterase; MBP, myelin basic protein; TCL, T cell line.

Myelin basic protein was prepared as previously described (17). The peptides Flu-HA<sub>306-318</sub> (PKYVKQNTLKLAT) and tetanus<sub>830-843</sub> (QYI-KANSKFIGITQL) were synthesized by continuous flow, solid phase peptide synthesis on the basis of the F-moc/Bu<sup>t</sup> strategy. Peptides were purified by HPLC, and their identities were tested using ion spray mass spectrometry.

The TCL were generated by an IL-7-modified primary proliferation assay, a method that allows the rapid expansion of Ag-specific T cells, including in vivo activated cells. Briefly, PBMC were isolated from fresh leukophereses by Ficoll density gradients and were seeded in 96-well U-bottom plates in T cell medium (IMDM, Life Technologies, Grand Islands, NY) containing 2 mM L-glutamine, 50 µg/ml gentamicin, 100 U/ml penicillin/streptomycin (all from BioWhittaker, Walkersville, MD), and 5% pooled human plasma at  $1 \times 10^5$  cells/well, with addition of 10 ng/ml IL-7 (recombinant human IL-7; PeproTech, Rocky Hill, NJ). After 7 days (37°C and 5% CO<sub>2</sub>), cultures were split by transferring 100 µl of each cell culture into a daughter plate, which was pulsed with [<sup>3</sup>H]thymidine (Amersham, Arlington Heights, IL) at 1 µCi/well. The incorporated radioactivity (counts per minute) was measured by scintillation counting (Betaplate, Pharmacia LKB, Piscataway, NJ) 8 h later. Proliferation of cultures with Ag (25 µg/ml MBP or 5 µg/ml peptides) was compared with proliferation of negative control wells seeded without Ag. Positive cultures (stimulation index >2 and absolute counts per minute at least 3 SDs above the average counts per minute of negative control wells) were identified on the mother plates and were periodically restimulated. The Ag specificity was confirmed at the end of the second in vitro stimulation cycle in 48-h proliferation assays as previously described (18). All blood samples were collected according to an institutional review board-approved protocol, and informed consent was obtained before the study. None of the patients received any immunomodulatory or immunosuppressive treatment within 1 mo before blood collection.

#### Effects of PDE-4 and PDE-3 inhibitors on Ag-specific TCL proliferation and functional Ag avidity

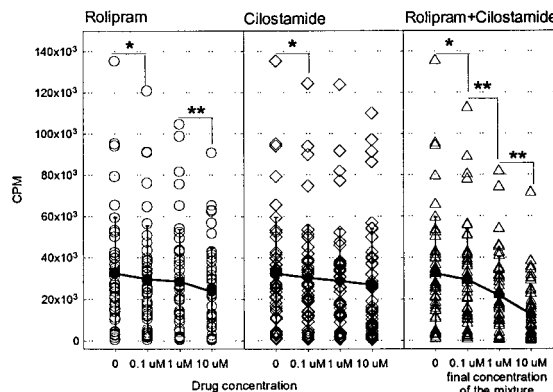
The effects of PDE inhibitors on Ag-specific proliferation, cytokine production, and functional Ag avidity were assessed during the third stimulation cycle (days 22–25 ex vivo). Each drug was used alone (in three concentrations: 0.1, 1, and 10 µM) or in combination (0.05, 0.5, and 5 µM, concentrations of individual drugs in the mixture). The selected dose range included the concentrations achievable in vivo (for rolipram, 0.09 and 0.2 µM; Dr. Claus-Steffen Stuerzebecher, Schering, Berlin, Germany, personal communication). Each condition was tested in duplicate, including negative (no Ag) and positive (Ag, no drug) controls. T cells were plated in 96-well U-bottom plates at  $2 \times 10^4$  T cells/well with irradiated autologous PBMC at  $1 \times 10^5$  cells/well. The Ag was added at the seeding concentration (25 µg/ml MBP and 5 µg/ml peptides) or over a wide range of Ag concentrations for the functional Ag avidity assay. Supernatants from these assays were collected after 36 h of incubation and stored frozen until analysis. For the last 8 h of incubation, cells were pulsed with [<sup>3</sup>H]thymidine at 1 µCi/well, and the incorporated radioactivity was measured by scintillation counting.

#### Cytokine secretion

Secretion of a Th1 (IFN-γ) and a Th2 (IL-4) cytokine by Ag-specific TCL was assessed by sandwich ELISA (Cyto-Sets from BioSource International, Camarillo, CA) according to the manufacturer's recommendation. All standards and samples were tested in duplicate.

#### Flow cytometry (FACS) analysis of the surface expression of costimulatory molecules

Fresh PBMC ( $1.2 \times 10^6$  cells/ml) were seeded in bulk cultures in 48-well plate with or without rolipram (10 µM). In addition to nonstimulated cells, the effect of rolipram was assessed upon nonspecific stimulation with LPS (2.5 µg/ml) or PHA-P (PHA; 5 µg/ml; both from Sigma). After 12-h incubation cells were washed with wash buffer (Dulbecco's PBS with 1% heat-inactivated FCS and 0.1% sodium azide) and incubated with fluorescein-, PE-, or Cy-Chrome-conjugated Ab (HLA-DR, DP, DQ-FITC, CD19-FITC, CD14-FITC and -PE, CD80-FITC and -PE, CD86-FITC and -PE, and CD3-Cy-Chrome; all from PharMingen, San Diego, CA) at saturating concentrations for 30 min on ice, then washed three times and analyzed (FACScan, Becton Dickinson, CA) using Cell-Quest software. Isotype-matched mouse IgG negative controls were used for each staining. Monocytes were gated based on the size characteristics (forward and side scatter), and expression of CD14. Lymphocytes were identified by the size characteristics and differentiation between T and B lymphocytes was based



**FIGURE 1.** Effect of PDE inhibitors on Ag-specific proliferation of TCL. A total of 47 TCL was stimulated with specific Ag without or with three increasing concentrations of rolipram and cilostamide. For the combination of rolipram and cilostamide, each drug was used in concentrations of 0.05, 0.5, and 5 µM, respectively. Results are depicted as counts per minute. Statistically significant differences are indicated (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). Only rolipram and the combination of both drugs had a dose-related inhibitory effect on Ag-specific TCL proliferation. The combination of rolipram and cilostamide was the most potent, demonstrating a synergistic effect of individual drugs. The differences among three treatment modalities reached statistical significance ( $p < 0.05$ , by repeated measures ANOVA analysis).

on the expression of CD3 and CD19 molecules, respectively. Five thousand cells in the gated population were analyzed per sample.

#### Statistical analysis

The data were analyzed by a commercial software package (Sigma-Stat, SPSS, Chicago, IL). The effects of the drugs on biological functions of TCL was evaluated by one-way repeated measures ANOVA or, if normality failed, by Friedman's repeated measure analysis on ranks. Statistically significant differences from repeated measures ANOVA were further analyzed by the Student-Newman-Keuls test, with  $p < 0.05$  as a cut-off for statistical significance. The effect of rolipram on costimulatory molecules was assessed by the Mann-Whitney rank-sum test.

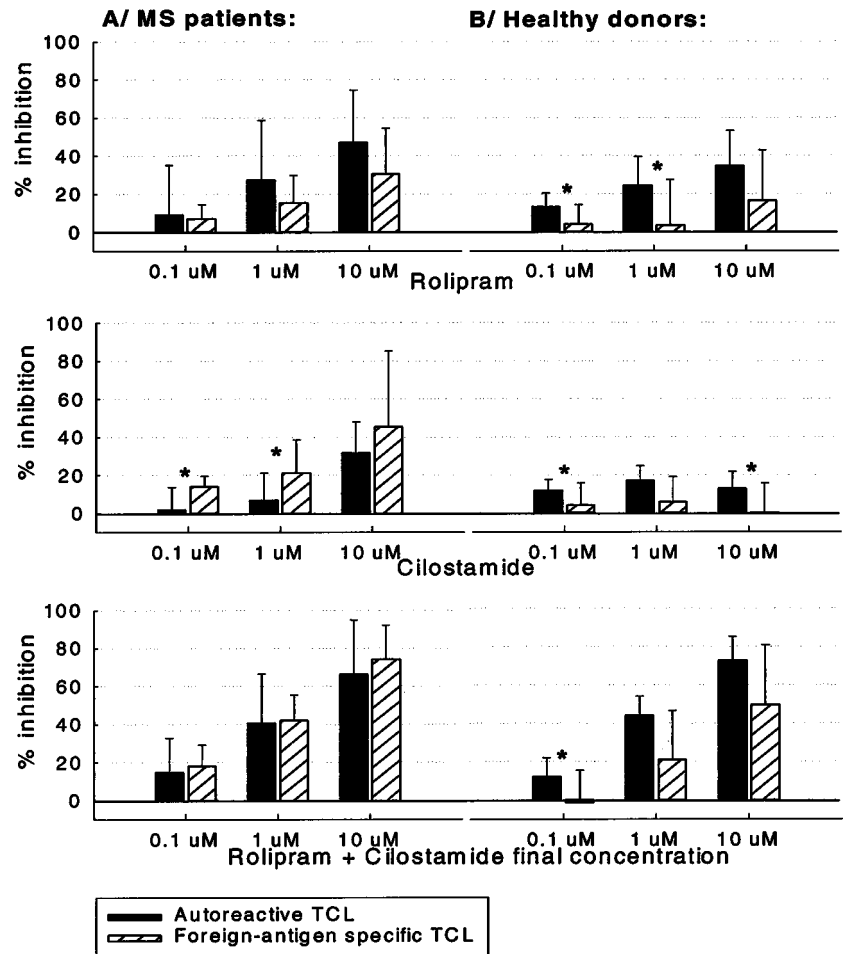
## Results

#### Effect of PDE-4 and PDE-3 inhibitors on Ag-specific TCL proliferation

We examined the effects of rolipram and cilostamide on the proliferation of 47 Ag-specific TCL. Both drugs inhibited Ag-driven TCL proliferation, but only rolipram and the combination of both drugs expressed statistically significant inhibitory effects in a dose-dependent manner (Fig. 1). The inhibition by rolipram (8.8–32% inhibition) was more pronounced than that by cilostamide (6.31–22.61% inhibition). Combinations of both drugs (1/1 ratio and half molar concentration of each drug (0.05, 0.5, and 5 µM in the final mixture) proved to be most efficient in inhibiting TCL proliferation (10.34–61.05%), exceeding the additive effects of individual drugs. The observed differences between drugs and their combination were statistically significant ( $p < 0.05$ , by repeated measures ANOVA). Because the above characteristics of the drug combination indicated synergistic effects, we have quantified the degree of synergism between rolipram based on the modified Berenbaum equation (19):

The degree of synergism between drugs A and B is equal to  $1/R$ , where  $R = [(IC_{20} \text{ drug A} + B)/(IC_{20} \text{ drug A})] + [(IC_{20} \text{ drug B} + A)/(IC_{20} \text{ drug B})]$ .

Based on the proliferation data in Fig. 1 we have estimated the  $IC_{20}$  (the concentration of each drug that leads to 20% inhibition of TCL proliferation; this was substituted for  $IC_{50}$ , as neither rolipram nor cilostamide reached 50% of inhibition) from the dose-response curves and calculated  $R_{\text{rolipram+cilostamide}} = 0.1123$ . The



**FIGURE 2.** Effects of PDE inhibitors on proliferation of TCL stratified based on their origin (derived from MS patients vs healthy donors) and selecting Ag (autoreactive vs foreign-Ag specific). Upon stratification of the proliferation data with respect to the origin of the TCL and the type of selecting Ag, a hierarchy of the sensitivity to PDE inhibition emerged; MS-derived TCL were more sensitive than healthy donor (HD)-derived TCL. A similar differential sensitivity to PDE4 vs PDE3 inhibition was observed in the analysis of autoreactive and foreign Ag-specific TCL within each population group. Statistically significant differences are indicated ( $p < 0.05$ , by Friedman's repeated measures analysis on ranks).

degree of synergism between rolipram and cilostamide is  $1/R = 8.9$ . (Values  $< 1$  indicate antagonism, 1 indicates additivity, and values  $> 1$  indicate synergism.)

*Comparison of the susceptibility to PDE-4 and -3 inhibition by autoreactive and foreign Ag-specific TCL and by TCL derived from MS patients and healthy donors*

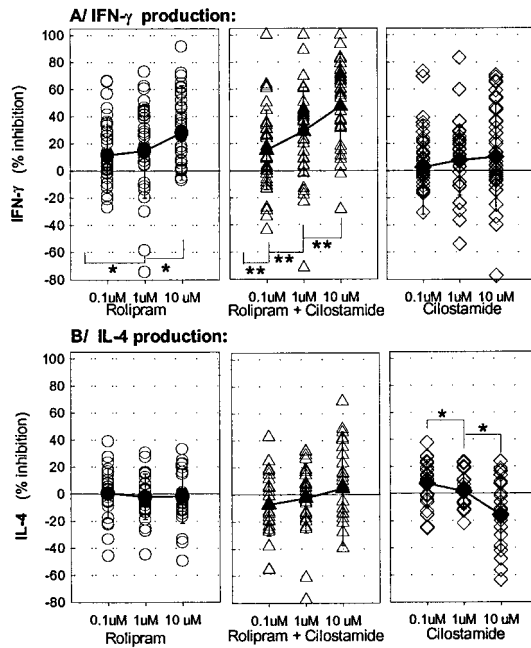
When the inhibitory effect of PDE inhibitors was assessed after stratification of TCL based on their origin and the type of the selecting Ag (TCL derived from MS patients (26 TCL, 17 autoreactive, 9 foreign-Ag-specific) vs. TCL derived from healthy donors (21 TCL, 5 autoreactive, 16 foreign-Ag-specific)), two interesting observations emerged (Fig. 2). 1) The MS-derived TCL were more susceptible to the effect of PDE inhibitors than were the healthy donor-derived TCL ( $p < 0.05$  for 10  $\mu\text{M}$  rolipram, 10  $\mu\text{M}$  cilostamide, and 0.1  $\mu\text{M}$  and 1  $\mu\text{M}$  concentrations of drug mixture; Friedman's repeated measures analysis on ranks). 2) Also, there appeared to be a differential sensitivity of autoreactive TCL and foreign Ag-specific TCL to the effects of PDE4 and PDE3 inhibition. Autoreactive TCL were inhibited by rolipram to a greater extent than foreign Ag-specific TCL ( $p < 0.05$ , through the tested concentration range of rolipram, Friedman's repeated measures analysis on ranks). Only stratified data are depicted in Fig. 2. Due to the decreased power of a comparison of divided data into individual patient groups, not all differences reached statistical significance ( $p < 0.05$ , by Friedman's repeated measures analysis on ranks); these are marked with an asterisk.

*Effect of PDE inhibitors on cytokine production by Ag-specific TCL*

We evaluated the effects of PDE inhibitors on Ag-driven production of two cytokines, IFN- $\gamma$  (prototypic Th1 cytokine) and IL-4 (prototypic Th2 cytokine; Fig. 3). Although a significant variability was noted among individual TCL, the average effect of PDE4 inhibition by rolipram and of PDE4 and PDE3 inhibition by the combination of drugs on IFN- $\gamma$  production was inhibitory. Rolipram (12.26–27.8% inhibition) and the combination of both drugs (16.07–46.3%) inhibited IFN- $\gamma$  synthesis in a dose-dependent manner. The effect of cilostamide was mild and did not reach statistical significance for any concentration (4.47–11.27% inhibition). Differences among the drugs were again statistically significant.

Neither rolipram nor the combination of rolipram and cilostamide had a statistically significant effect on IL-4 production (inhibition ranging from  $-0.62$  to 1.69% throughout the concentration range). Cilostamide had a mild inhibitory effect on IL-4 production at 0.1  $\mu\text{M}$ , whereas a high concentration (10  $\mu\text{M}$ ) significantly enhanced IL-4 production ( $-11.39\%$  inhibition at 10  $\mu\text{M}$  concentration). Overall, the effect of PDE4 and PDE3 inhibitors on IFN- $\gamma$  production paralleled those on proliferation, with the drug combination having synergistic effects. The IL-4 production was largely unaffected despite the demonstrated significant inhibition of TCL proliferation at the drug concentrations tested.





**FIGURE 3.** Effect of PDE inhibitors on cytokine production of Ag-specific TCL. The effects of rolipram, cilostamide and their combination on Ag-specific production of IFN- $\gamma$  and IL-4 was assessed in parallel to the proliferation. Results are depicted as the percent inhibition of cytokine production for each concentration of drug compared with control cultures (no drug added). Statistically significant differences are indicated (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). Although all TCL tested in proliferation assays produced IFN- $\gamma$  and are included in the analysis, only 9 of 26 MS-derived TCL and 17 of 21 healthy donor-derived TCL produced IL-4; therefore, only these 26 TCL could be included in the analysis of the effect of PDE inhibitors on IL-4 production. Only rolipram and the combination of rolipram with cilostamide inhibited the production of IFN- $\gamma$  significantly compared with control samples. This inhibition paralleled the effect on TCL proliferation and was clearly strongest for the combination of both drugs. Neither rolipram nor the drug combination has a significant effect on IL-4 production. A high concentration (10  $\mu$ M) of cilostamide enhanced IL-4 production despite the demonstrated inhibitory effect on proliferation. Differences between individual drugs were statistically significant ( $p < 0.05$ ).

#### Effect of PDE inhibitors on the functional Ag avidity of TCL

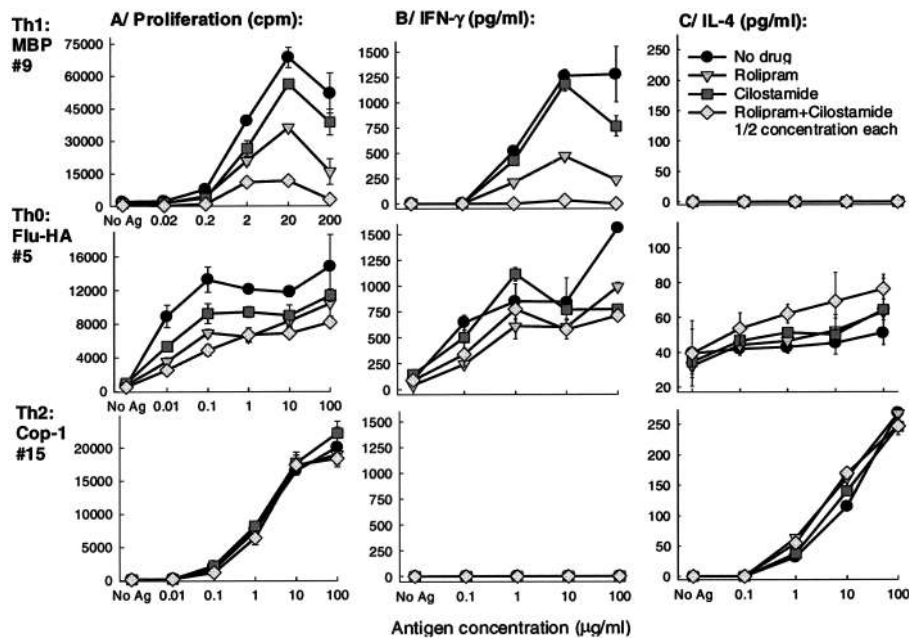
To gain a better understanding of the mechanism of the PDE4 and PDE3 inhibitor-induced bias of immune responses from a Th1 toward a Th2 phenotype, we decided to study their effects on the major components of T cell priming. Apart from the influence on the cytokine milieu, which was supported by the above experiments, we wanted to address the question of whether these drugs influence the Ag dose needed for T cell activation, i.e., antigen avidity, and costimulatory signals delivered by APC. First, to address the question of whether the magnitude of the immunomodulatory effect of PDE inhibitors on TCL varies depending on the dose of Ag, we exposed a subgroup of TCL (15 TCL; selected as a representative sample based on their phenotype, variable susceptibility to PDE inhibition, and reactivity to either autoantigen or environmental Ag; Table I) to a 1- $\mu$ M concentration of the individual drugs over a wide range of Ag concentrations. The results of this functional Ag avidity assay for three TCL (of Th1, Th0, and Th2 phenotypes) are summarized in Fig. 4. None of the autoreactive or foreign Ag-specific TCL that we generated for this project expressed a clear Th2 phenotype. We therefore included a Th2-like TCL specific for copolymer-1 (Cop-1), an approved immunomodulatory drug for MS. The TCL Th1:MBP and Th0:Flu-HA were representative of all other tested TCL and illustrate the above-mentioned differential effect of PDE inhibition on autoreactive vs foreign Ag-reactive TCL. All examples also demonstrate the hierarchy of immunomodulation between individual drugs (cilostamide < rolipram < rolipram + cilostamide). For MBP-specific Th1-TCL, a combination of PDE4 and PDE3 inhibition resulted in almost 100% inhibition of proliferation and IFN- $\gamma$  production. For Flu-HA-specific Th0-TCL, the inhibition of proliferation and IFN- $\gamma$  production was much less pronounced (~50% with the combination of drugs for each Ag concentration), and the production of IL-4 was either unaffected or even enhanced. For Cop-1-specific Th2-TCL, the individual drugs had no effect on Ag-specific proliferation or IL-4 production. Overall, the magnitude of the effect of PDE inhibitors on individual TCL was similar through the tested Ag concentration range. However, as demonstrated in Table I, the PDE inhibition had a mild, but statistically significant, effect on the  $EC_{50}$  (concentration of Ag that leads to 50% maximal proliferation) of individual TCL ( $p = 0.009$ , Friedman's repeated measures analysis on ranks).

Table I. Effect of PDE inhibitors on TCL Ag avidity ( $EC_{50}$ )

TCL	Ag	TCL Origin	Phenotype	$EC_{50}$ ( $\mu$ g/ml)			
				No drug	Cilostamide	Rolipram	Rolipram + cilostamide
1	Flu-HA	MS	Th1	0.070	0.070	0.080	0.200
2	Flu-HA	MS	Th1	0.070	0.200	0.200	0.200
3	Flu-HA	MS	Th1	0.009	0.010	0.030	0.500
4	Flu-HA	MS	Th0	0.003	0.003	0.100	0.100
5	Flu-HA	MS	Th0	0.008	0.010	0.100	0.100
6	MBP	MS	Th1	0.050	0.070	0.070	0.100
7	MBP	MS	Th1	10.000	10.000	— <sup>a</sup>	— <sup>a</sup>
8	MBP	MS	Th1	2.000	3.000	2.000	3.000
9	MBP	MS	Th1	1.500	1.500	1.500	1.400
10	MBP	HD	Th1	20.000	20.000	20.000	30.000
11	MBP	MS	Th0	3.000	3.000	10.000	— <sup>a</sup>
12	MBP	MS	Th0	1.000	1.500	1.000	1.000
13	MBP	HD	Th0	25.000	25.000	25.000	25.000
14	MBP	HD	Th0	3.000	3.000	4.000	3.000
15	Cop-1	HD	Th2	3.000	3.000	3.000	3.000
Total	Mean $\pm$ SD			4.58 $\pm$ 7.77	4.69 $\pm$ 7.73*	4.79 $\pm$ 8.02*	5.20 $\pm$ 10.02*

<sup>a</sup> 100% inhibition of proliferation.

\*,  $p < 0.05$ ; Friedman's repeated measures analysis on ranks with Student-Newman-Keuls test.



**FIGURE 4.** Effect of PDE inhibitors on functional Ag avidity. A total of 15 TCL were tested in Ag dose-response assays in the presence of  $1 \mu\text{M}$  of drug and a wide Ag concentration range. Only three TCL are depicted in this figure. Th1:MBP (TCL 9 in Table I) and Th0:Flu-HA (TCL 5 in Table I) were selected as representative TCL for 14 MBP- or Flu-HA-specific TCL tested. The Th2:Cop-1 (TCL 15 in Table I) TCL was added for this assay only, as none of the 47 MBP- or Flu-HA-specific TCL tested in previous experiments expressed a clear Th2 phenotype. The  $\text{EC}_{50}$  (Ag concentration leading to 50% maximal proliferation of TCL) values were calculated from these dose-response curves for each TCL and are summarized in Table I. The TCL depicted in this figure demonstrate the hierarchy of potency of the immunomodulatory effect between individual drugs. The combination of rolipram and cilostamide had the strongest inhibitory effect on T cell proliferation and  $\text{IFN-}\gamma$  secretion, while having no effect or enhancing the production of IL-4. The differential susceptibility to PDE inhibitor-mediated immunomodulation on TCL was noted based on their cytokine phenotype, with Th1-TCL being more susceptible than Th0 or Th2-TCL. Overall, the magnitudes of the effects of PDE inhibitors on individual TCL were similar through the tested Ag concentration range.

#### Effect of rolipram on the surface expression of costimulatory molecules

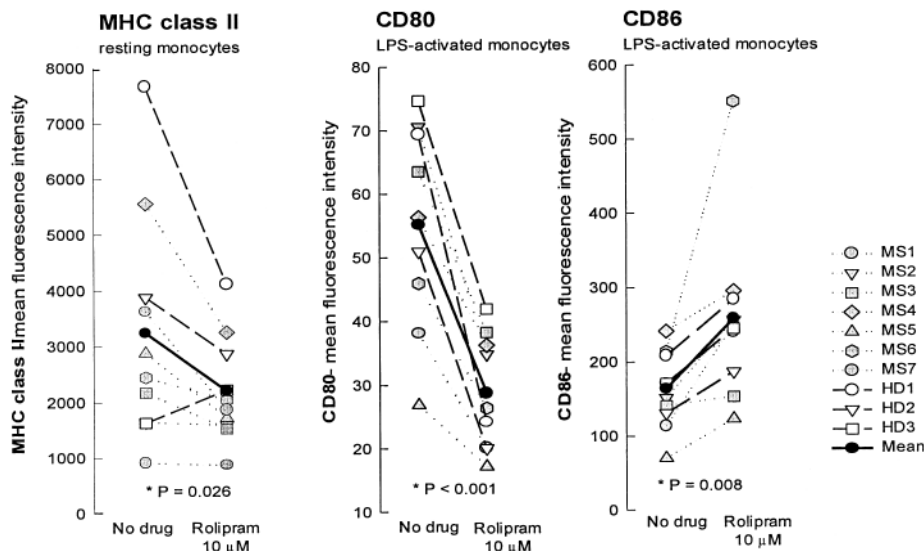
Finally, we wanted to assess the effect of PDE inhibition on the third important component of T cell priming, the costimulatory signal. We studied the effect of rolipram on the surface expression of costimulatory molecules of human PBMC in the resting and in the activated state. Freshly isolated PBMC were seeded *ex vivo* with or without rolipram, in a resting state or upon activation with nonspecific stimuli (PHA or LPS). After 12 h we analyzed these cells by three-color flow cytometry. Rolipram consistently down-regulated the surface expression of MHC class II both in the resting state and after stimulation with PHA and LPS on monocytes (Fig. 5) and T and B lymphocytes (data not shown). After 12-h stimulation of PBMC with LPS, rolipram-treated cultures had decreased surface expression of CD80 ( $p < 0.001$ , by Mann-Whitney rank-sum test) and increased surface expression of CD86 ( $p = 0.008$ ; Fig. 5). Similar changes in the expression of costimulatory molecules were observed on B and T lymphocytes or after stimulation with PHA (data not shown).

## Discussion

In this article we present a detailed analysis of the effects of the selective PDE4 inhibitor rolipram and the PDE3 inhibitor cilostamide on human immune cells to determine the potential of these drugs for the treatment of human Th1-mediated autoimmune disorders. Consistent with the data obtained from animal models (5, 10, 11), we demonstrated the predominant inhibitory effect of PDE inhibitors on Th1-mediated immune responses in humans. To address the effect of PDE inhibitors on the cytokine phenotype of human T cells, we focused our experiments on short term TCL and performed our analysis on days 22–25 *ex vivo* to avoid possible

artifacts of long term culture. Our data on the modulation of Th2-TCL by PDE inhibitors are limited, but in agreement with our cytokine data from Th0-TCL (no effect, or even induction of IL-4) and with data from different experimental systems (7, 20, 21); we found no inhibition of Ag-specific TCL proliferation and IL-4 production in these TCL.

Few studies have addressed the question of the effect of PDE inhibitors on human immune cells. A recent study from our laboratory examined the expression of PDE4 and PDE3 enzymes in autoreactive MBP-specific TCL (2) and demonstrated that these two families account for the vast majority of PDE enzymatic activity in these cells. This is consistent with our current data, demonstrating a significant inhibition of proliferation at the highest concentration of the drug combination ( $5 \mu\text{M}$  of both rolipram and cilostamide) for virtually all tested TCL. Another study analyzed the influence of rolipram on the functional characteristics of nine MBP-specific TCL, five derived from MS patients and four from healthy donors (15), and raised the issue of a differential susceptibility of individual TCC to the immunomodulatory influence of rolipram. Although rolipram inhibited  $\text{TNF-}\alpha$  and  $\text{-}\beta$  as well as IL-10 production by TCL, the effect on other cytokines ( $\text{IFN-}\gamma$ , IL-4, and IL-13) was inconsistent and did not reach statistical significance. Moreover, Essayan et al (16), suggested a higher susceptibility to PDE4 inhibition by Th2-TCL compared with Th1-TCL in a limited number of Th1 and Th2 TCL (total of four) derived from atopic and asthmatic patients. Although it is difficult to compare the data derived from different experimental systems, the reported inhibition of Th1-TCL derived from these asthmatic patients did not reach the magnitude of the typical inhibition of MS-derived Th1 TCL observed in our laboratory using the same concentrations of drugs. Because asthmatic individuals and MS



**FIGURE 5.** Effect of rolipram on the expression of costimulatory molecules by APC. The surface expression of MHC class II and costimulatory molecules CD80 and CD86 on monocytes and B and T lymphocytes was assessed by triple-staining, flow cytometric analysis. Resting or nonspecifically activated (LPS or PHA) PBMC were incubated for 12 h in bulk culture with or without rolipram and analyzed by flow cytometry. Only the effect on monocytes (gated based on size characteristics and CD14 expression), both rested and activated by LPS, is depicted in this figure, but similar results were obtained upon activation by PHA or when gating of B or T lymphocytes. Rolipram down-regulates MHC class II expression on both resting and activated cells, while it down-regulates CD80 and up-regulates CD86 expression upon activation with either nonspecific stimulus. These changes were observed in both MS patients and healthy donors.

patients have biased immune responses toward opposite Th phenotypes as compared with unbiased responses of healthy donors, we asked whether the explanation for these controversial data from human TCL lies in the differential susceptibility to PDE inhibition between these different patient groups. This hypothesis prompted us to examine the effect of PDE inhibition on large numbers of TCL, derived from both healthy donors and MS patients, and with specificity for two Ags, the autoantigen MBP and the classical foreign recall Ags Flu-HA or tetanus (22). Indeed, we were able to demonstrate a higher susceptibility to PDE inhibition by MS-derived compared with healthy donor-derived TCL. This finding has several important implications. It explains how the same therapeutic agent could be considered for the treatment of disorders with potentially different pathogenesis (Th1-mediated autoimmune disorders vs. Th2-mediated asthma and atopic dermatitis). We and others recently demonstrated (23, 48) that the immune system in MS patients is in a dysregulated state characterized by an overshooting Th1 response not only to autoantigens, but also to common environmental pathogens. A similar dysregulated state, this time toward Th2 responses, is likely to exist in asthmatic or atopic individuals (24). If such a dysregulation involves abnormalities in the cAMP second messenger system, it would render TCL derived from these individuals more susceptible to the effects of PDE inhibition, thus at least in part explaining the controversies between results obtained from MS and asthmatic patients. Several reports in the literature indicate that this may be the case. Patients with MS, rheumatoid arthritis, or lupus were found to have low intracellular cAMP levels (25, 26), decreased expression and activity of G protein-coupled receptor kinases (27), or deficient type I cAMP-dependent protein kinase A activity (28, 29). Similar abnormalities in cAMP signaling were suggested in asthmatic patients (30–32).

It is more difficult to explain the suggested differential susceptibility to PDE inhibition between autoreactive and foreign Ag-reactive TCL. We did not find any significant skewing in the cytokine profiles between these two types of TCL that would account for the observed differential effect. This effect is not mediated by

the need for antigenic processing of MBP compared with Flu-HA and tetanus peptides, because it was shown that MBP presentation by HLA-DR molecules does not require processing (33) and because we have noted a similar effect of PDE inhibition on seven MBP-specific TCL stimulated with the peptide epitope (data not shown). The two likely explanations are that either the signal delivered by the autoantigen may be qualitatively different (partial agonist vs. full agonist signal) or the dysregulation in the cAMP system is more pronounced in autoreactive T cells. We are currently studying this issue in detail. Considering the therapeutic use of PDE inhibitors, the observed higher susceptibility of autoreactive TCL to PDE4 inhibition may widen the therapeutic window in the treatment of autoimmune disorders without inducing general immunosuppression.

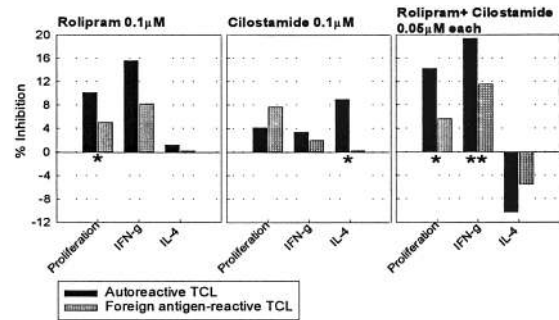
To explore the possible cause of the PDE inhibitor-induced bias from Th1 to Th2 phenotype, we decided to study the influence of these drugs on T cell priming. There are three major components contributing to T cell priming, which may influence the phenotype of the primed T cell: the cytokine milieu, the dose and character of the Ag, and the costimulatory signal. It was previously demonstrated by us and others that selective PDE4 inhibitors or nonselective PDE inhibitors decrease the secretion or expression of proinflammatory cytokines by human mononuclear cells, favoring the cytokine milieu at the time of Ag presentation toward an anti-inflammatory Th2 phenotype (6, 14, 34, 35). However, the influences of PDE inhibition on the other components of T cell priming conditions were unknown. First, we studied the PDE inhibitors in Ag dose-response assays, exploring their effects on the dose of Ag required for T cell activation. The results of these studies indicate that PDE inhibitors have two different effects on the activation of TCL; they inhibit TCL proliferation regardless of the Ag dose (Fig. 4). On the other hand, the effect of PDE inhibitors on the  $EC_{50}$  indicates that a higher Ag dose is necessary for the activation of TCL under their influence. These data suggest that PDE inhibitors have complex inhibitory effects on T cell activation, most likely by influencing both proximal Ag-responsive events of T cell signaling



(36) as well as components of more downstream machinery involved in T cell effector functions. The exact mechanisms of this effect are currently under investigation in our laboratory. The demonstrated influence of PDE4 and -3 on the Ag dose required for T cell activation together with the data from the literature showing that high Ag doses skew the developing immune response toward a Th1 phenotype, whereas low doses of Ag skew the response toward a Th2 phenotype (37) adds another mechanism for the observed effect of these drugs on Th1/Th2 paradigm.

Next, we wanted to assess the influence of rolipram on the third component of T cell priming, the costimulatory signals. It has been suggested that costimulation by CD80 preferentially drives the T cell differentiation toward Th1 responses, whereas CD86 costimulation biases T cell priming toward Th2 responses (38). Although some concerns were raised regarding the general validity of this dichotomy of the roles of CD80 and CD86 (39), several reports indicate that the CD80/CD86 costimulatory system is altered in MS patients. Specifically, higher numbers of CD80<sup>+</sup> B lymphocytes in the cerebrospinal fluid (40, 41) increased serum levels of CD80<sup>+</sup> lymphocytes in patients during MS exacerbation (42), and low expression of CD86 on cerebrospinal fluid T cells (41) have been reported in patients with MS. Our data indicate that rolipram down-regulates CD80 expression and up-regulates CD86 expression on monocytes and B and T lymphocytes upon nonspecific activation with PHA or LPS. The likely explanation for this observation is the differential kinetic of induction of these costimulatory molecules on APC; CD80 is expressed later than CD86; therefore, rolipram may be preventing the switch from CD86 to CD80 expression, an issue that merits further study. We also demonstrated that rolipram down-regulates MHC class II expression, both in resting conditions and after induction by proinflammatory signals. This finding is in agreement with the observation in a murine system, where increases in intracellular cAMP inhibit the IFN- $\gamma$ -mediated induction of class II MHC genes (43). Together, these changes in the costimulatory profile on APC favor T cell priming from Th1 toward a Th0 or Th2 phenotype and may limit the effective presentation of autoantigen in inflammatory MS lesions. Indeed, rolipram was shown to reduce the number of IFN- $\gamma$ -secreting cells upon priming of human mononuclear cells in bulk cultures to the autoantigen MBP, while the numbers of IL-4- or IL-10-secreting cells were unaffected (44).

The above data demonstrate a favorable drug profile of PDE4 and PDE4 combined with PDE3 inhibitors for the treatment of Th1-mediated autoimmune disorders; however, the question remains of whether this immunomodulatory effect is expressed within a concentration range that is achievable in humans in vivo. The concentrations of rolipram achievable in healthy volunteers following the administration of 0.75 mg three times daily and 1.5 mg three times daily were 24 and 53 ng/ml (0.09–0.2  $\mu$ M; Dr. Claus-Steffen Stuerzebecher, unpublished observations) Our data, summarized in Fig. 6, indicate that rolipram and especially the combination of rolipram and cilostamide have a mild immunomodulatory effect at the concentration of 0.1  $\mu$ M. However, due to the immunomodulation at multiple levels (influence on T cell priming conditions, Ag-specific proliferation, and cytokine production) the in vivo effect is likely to be more prominent. The combination of PDE4 and PDE3 inhibitors expresses a high degree of synergism that is, to our experience, achievable even at much lower concentrations of cilostamide in the final mixture (data not shown). Therefore, the combination of PDE4 inhibitors with relatively small amounts of PDE3 inhibitors may represent a way to broaden the therapeutic window in the treatment of human disorders and should be considered for future drug development. The molecular mechanism of this synergy is not known. It has been



**FIGURE 6.** Immunomodulatory effect of PDE inhibitors within in vivo achievable concentration ranges. The concentration of rolipram that can be achieved in humans in steady state without major side effects ranges from 0.09 to 0.2  $\mu$ M. At a concentration of 0.1  $\mu$ M, PDE inhibitors express a mild, but multilevel, immunomodulatory profile by suppressing Ag-specific proliferation and IFN- $\gamma$  production preferentially of autoreactive TCL, while not affecting, or even enhancing, IL-4 production. This immunomodulation is strongest for the combination of rolipram and cilostamide, demonstrating the synergistic effect. A statistically significant effect compared with control cultures is indicated (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

reported that PDE3 inhibitors alone have little effect on the total intracellular cAMP levels, and they do not further enhance the cAMP accumulation induced by rolipram (45). However, it has been suggested, that PDE3 (predominantly localized to the particulate cellular fraction) and PDE4 (predominantly cytosolic) may regulate different pools of cAMP (46, 47). It is conceivable that intracellular signaling can partially adapt to the effects of PDE4 inhibition by diverting critical pathways blocked by high cytosolic cAMP concentrations and activation of PKA to the alternative pathways, which, in turn, may be affected by PDE3 inhibition. Elucidating the molecular mechanism of this synergy between PDE4 and PDE3 inhibition will enhance our understanding of cAMP second messenger signaling.

Well-designed therapeutic trials supported by immunological studies should not only provide more definite information about the therapeutic potential of PDE inhibitors in autoimmune disorders, but also broaden our understanding of the immunopathogenesis and the potential differences among individual human Th1-mediated autoimmune diseases.

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