

Phosphodiesterase profiles of highly purified human peripheral blood leukocyte populations from normal and atopic individuals: A comparative study

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Background: Several previous reports have suggested an increased activity of cAMP phosphodiesterases (PDEs) and a higher sensitivity of these enzymes toward PDE inhibitors in leukocytes of patients suffering from atopic dermatitis. **Objective:** The purpose of the present study was to comprehensively analyze and compare the PDE expression and activity profile of highly purified populations of leukocytes from normal and atopic blood donors. In addition, the influence of PDE inhibitors on function of leukocytes from normal and atopic individuals was investigated. **Methods:** Density gradient centrifugation, elutriation, and magnetic cell sorting techniques were used to purify eosinophils, monocytes, and B and T lymphocytes from peripheral human blood. Complementary DNA-polymerase chain reaction was used to analyze PDE4 subtype messenger RNA (mRNA) expression levels in addition to PDE isoenzyme activities. PDE4 inhibitor sensitivity was determined in monocyte homogenates from both groups. Functionally, suppression of lipopolysaccharide-induced synthesis of tumor necrosis factor- α in monocytes as well as phytohemagglutinin-induced T cell proliferation in peripheral blood mononuclear cell fractions by PDE4 and PDE3/4 inhibitors was compared. **Results:** Identical PDE activities and mRNA expression profiles were found in all cells from normal and atopic donors except that there was an increase in the mRNA levels of PDE4A and PDE4B2 in atopic T cells, which was, however, not reflected in overall PDE4 activity. In addition, no differences in sensitivity of the functional responses to PDE inhibitors were noted. The mixed PDE3/4 inhibitor zardaverine was a more potent inhibitor of T cell proliferation than rolipram, a selective PDE4 inhibitor. **Conclusion:** No evidence for alterations of PDE activities in atopy is provided by our findings. (*J Allergy Clin Immunol* 1997;100:527-35.)

Key words: Phosphodiesterases, phosphodiesterase 4 subtypes, atopic dermatitis, rolipram, zardaverine, RS-25344, T cell proliferation, cyclic 3',5' adenosine monophosphate, tumor necrosis factor- α

Abbreviations used

β 2m:	β 2 microglobulin
$\text{Ca}^{2+}/\text{CaM}$:	Calcium/calmodulin
cAMP:	Cyclic 3', 5' adenosine monophosphate
cGMP:	Cyclic 3', 5' guanosine monophosphate
FCS:	Fetal calf serum
LPS:	Lipopolysaccharide
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
PDE:	Phosphodiesterase
PHA:	Phytohemagglutinin
PMN:	Polymorphonuclear granulocyte
TNF:	Tumor necrosis factor- α

Cyclic nucleotide phosphodiesterases (PDEs) are classified in seven distinct families based on genetic structure, kinetic parameters (substrate specificity and affinity), and regulation by specific activators or inhibitors. By their hydrolyzing activity, these enzymes represent key regulators of intracellular cyclic 3', 5' adenosine monophosphate (cAMP) and cyclic 3', 5' guanosine monophosphate (cGMP) levels, thus controlling a large variety of cellular responses that are elicited by these two second messengers. In inflammatory cells, subtypes of the low K_m cAMP-specific PDE4 are predominant in terms of expression and function. PDE4 is believed to play an important immunoregulatory role, because many inflammatory reactions are decreased in the presence of PDE4-selective inhibitors such as rolipram.¹⁻³ During the last two decades, several reports have pointed to a higher PDE4 activity in leukocytes of atopic patients. Increased PDE4 activities have been described in mononuclear fractions of atopic blood cells^{4,5} and an atopy-specific, calcium/calmodulin ($\text{Ca}^{2+}/\text{CaM}$)-dependent cAMP PDE has been immunochemically characterized by Chan et al.⁶ In addition to atopy-specific PDE enzyme expression or increased PDE activity in atopic cells, a diminished proliferative response toward a T cell stimulus^{7,8,9} and a higher sensitivity towards PDE inhibitors¹⁰ have been reported in PHA-stimulated peripheral blood mononuclear cells (PBMCs) from atopic patients. These alterations in PDE activities, which in turn

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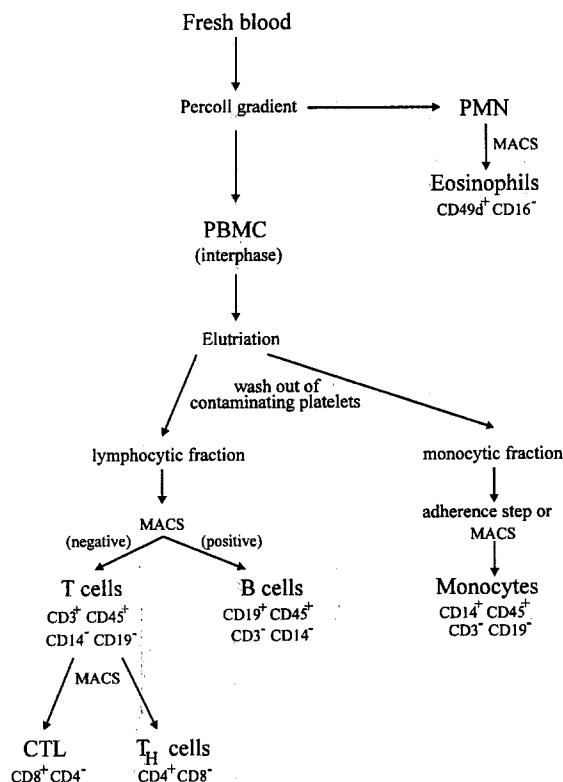


FIG. 1. Schematic overview of cell purification steps.

should lead to an increased breakdown of cAMP, are believed to cause abnormalities in immune functions, which finally contribute to atopy. From a pharmacologic point of view, the existence of an atopy-specific PDE4 variant would be extremely promising. First, the development of atopy-selective PDE inhibitors could be approached. Second, characterization and functional analysis of such an enzyme could provide new insights and hints to the underlying causes of the unbalanced immunologic responses seen in patients suffering from atopic dermatitis and atopic asthma.

Theoretically, increased PDE4 activity in atopic leukocytes could also be due to different regulation of the same (i.e., "normal") PDE4 enzyme(s), and the enzyme therefore does not necessarily have to be a new PDE variant. The activation status of PDE4 is both transcriptionally and postranscriptionally regulated. For instance, the activity of certain PDE4 subtypes can be increased by either a short-term regulatory process involving protein phosphorylation¹¹⁻¹⁴ or a long-term regulatory process involving increased gene expression within hours or days.^{15, 16} Either of these regulatory mechanisms is dependent on a cAMP signal and can be triggered by a variety of adenyl cyclase activators.^{11, 17, 18} This raises the possibility that PDE4 activity in vivo is subject to regulation by hormones, growth factors, and drugs. Consequently, changes in PDE4 activity in leukocytes from atopic individuals could simply result from an imbalance in the cellular environment.

Many studies on PDE isoenzyme distribution and on functional consequences of PDE inhibition in leukocytes have been performed in mixed cell populations such as PBMCs. In this study we wanted to clarify (1) whether differences exist in the PDE profiles of highly purified populations of inflammatory cells (relevant in asthmatic and atopic disorders) from normal and atopic individuals, (2) whether inhibition constants for well-characterized PDE4 inhibitors differ, and (3) whether these putative differences are reflected functionally for certain leukocyte responses in vitro.

METHODS

Blood donors

Normal healthy donors who had no history of asthma, allergic rhinitis, or atopic dermatitis were selected for this study and compared to a separate group of individuals suffering from atopic dermatitis whose only medications were corticosteroids. The severity of the disease was clinically assessed as mild (minor skin symptoms and low IgE levels) or severe (obvious skin lesions and IgE levels generally higher than 1000 IU/ml). All patients had stopped taking medication for at least 2 weeks before the investigation. This study was approved by the local ethics authorities and was carried out according to national guidelines.

Purification of leukocytes

Fig. 1 schematically summarizes the isolation protocol, which is outlined in detail below.

Eosinophils. Eosinophils were purified essentially as described by Hatzelmann et al.¹⁹ Peripheral venous blood was obtained from normal and atopic donors and was anticoagulated with heparin (10 U/ml). Blood was diluted with an equal volume of 2% heat-inactivated fetal calf serum (FCS) in phosphate-buffered saline (PBS) (PBS/FCS). Diluted blood was layered over 18 ml of isotonic Percoll (density, 1.082 gm/ml) and centrifuged at 600 g for 30 minutes at 25°C. After centrifugation, the upper layer containing mononuclear cells was discarded, and the red cell pellet containing the polymorphonuclear granulocyte (PMN) fraction was removed before erythrocyte lysis (15 minutes at 4°C) with a buffer containing NH_4Cl (155 mmol/L), KHCO_3 (10 mmol/L), and ethylenediaminetetraacetic acid (0.1 mmol/L) at pH 7.4. PMNs were then washed twice in PBS/FCS and finally resuspended in 500 μl of PBS/FCS. For eosinophil purification, neutrophils were depleted by using magnetic antibodies directed against CD16 (30-minute incubation on ice) on a MACS-column type C (Miltenyi Biotec, Bergisch-Gladbach, Germany) placed in a strong magnetic field. Unlabeled eosinophils (95% to 97% purity) were eluted from the column with 25 ml of PBS/FCS. To further increase eosinophil purity, the immunomagnetic procedure was repeated, and eosinophil purity attained was greater than 99.5% as assessed by Kimura staining.

Monocytes and lymphocytes. For each experiment 250 ml of peripheral venous blood was drawn from a single volunteer. Citrate (0.31%) or heparin (10 U/ml) was used as an anticoagulant. The blood was diluted 1.6-fold with PBS (pH 7.4) before centrifugation at 220 g at 20°C for 20 minutes. The cell pellet was layered on a Percoll gradient (density, 1.077 gm/ml), and the interphase containing PBMCs was obtained after a 800 g centrifugation at 20°C for 25 minutes. Cells were washed twice in elutriation medium (PBS, 2% heat-inactivated human AB serum, 2 mmol/L EDTA, 5 mmol/L glucose, pH 7.4) before

countercurrent centrifugal elutriation of the cells in a J2-MC centrifuge equipped with a JE-6B rotor (Beckman).

The lymphocyte-containing fraction (platelet-free and of more than 95% purity) was obtained at a flow rate of 29 ml/min and a rotor speed of 3000 rpm. Pure B lymphocytes were obtained by positive selection by use of magnetic beads conjugated to anti-CD19 antibody and detaching antibody according to the manufacturer's protocol (Dynal, Oslo, Norway). The purity of B cells (CD19⁺ cells) was checked by using a flow cytometer (Coulter, Hamburg, Germany) and was always greater than 99%. No CD3⁺ T cells or CD14⁺ monocytes were detectable, which is in accordance with the unresponsiveness of the cells to the T cell mitogen phytohemagglutinin (PHA; data not shown). T cells were obtained by negative selection of the whole lymphocyte fraction on a MACS-column type C by using magnetic antibodies directed against CD19, CD14, and CD16 in order to deplete B cells, monocytes, and granulocytes/NK cells, respectively. Additional incubation of the cells with magnetic anti-CD4 or anti-CD8 antibodies was performed in order to obtain CD8⁺ cytotoxic T cells or CD4⁺ T helper cells, respectively.

The monocyte-containing fraction (platelet-free and 75% to 85% pure) was obtained at a flow rate of 39 ml/min at a rotor speed of 3000 rpm. These cells were further purified by depleting lymphocytes, granulocytes, and NK cells with antibody-coated magnetic bead (antibodies directed against CD3, CD19, and CD16, respectively).

Each individual leukocyte population was more than 99% pure as checked by flow cytometry (Coulter). All cell fractions were washed twice in PBS/FCS before further use.

PDE activity assay

Freshly prepared cells were disrupted by sonication in homogenization buffer (PBS, 10 mmol/L HEPES, pH 8.2, 1 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L β-mercaptoethanol, 1 mmol/L pepablock, 5 μmol/L pepstatin A, 10 μmol/L leupeptin, 10 μmol/L trypsin inhibitor, and 2 mmol/L benzamidine). The homogenate was spun at 1000 g before the supernatant was further separated by a 100,000 g centrifugation step (1 hour, 4°C). The supernatant was decanted (soluble fraction) and the remaining pellet was resuspended in an equal volume of buffer (particulate fraction). PDE activity was determined as described by Thompson et al.²⁰ with some modifications.²¹ In brief, the enzyme-containing fractions were assayed in a final volume of 200 μl containing 40 mmol/L Tris HCl, pH 7.4, 5 mmol/L MgCl₂, 0.5 μmol/L cAMP or cGMP (approximately 25,000 cpm of tritium-labeled cAMP or [³H] cGMP) and were incubated in the presence or absence of activators or inhibitors for 30 minutes at 37°C. The reaction was terminated by the addition of 50 μl of 0.2 N HCl, and the assay mixture was left on ice for a further 15 minutes. *Crotalus atrox* snake venom (0.5 mg/ml) was added for 15 minutes at 37°C and the assay mixture was loaded onto QAE-Sephadex A-25 columns (1 ml bed volume) and eluted with 2 ml ammonium formate (30 mmol/L, pH 6.0). The radioactivity in the eluate was counted in a liquid scintillation counter. PDE isoenzyme activity calculations in the soluble as well as in the particulate cell fractions were performed by using specific activators and the PDE family-selective inhibitors motapizone (for PDE3) and rolipram (for PDE4) as described previously.²²

Expression analysis by complementary DNA-polymerase chain reaction

Preparation of RNA, conditions for complementary DNA (cDNA)-polymerase chain reaction (PCR) with the thermocycler 60/2 from Bio-med (Theres, Germany), and separation of

PCR products by polyacrylamide gel electrophoresis were performed as described in previous studies.^{23, 24} The buffer for PCR provided by the supplier (Amersham) was applied strictly as recommended. After staining of the gels with ethidium bromide, the signals were directly digitalized by the CS-1 video-imager (Cybertech, Berlin, Germany). Signal intensities, calculated by using the WINCAM densitometric software (Cybertech), were assigned to the various genes of interest with reference to the β2-microglobulin (β2m) gene obtained by a simultaneously performed PCR on an aliquot of the same cDNA. The PDE4 isoforms detected and amplimers used were the following: (1) PDE4A: sense—TCAGAGCTGGCGCT-TATGTAC (adopted from reference 25, primer PE21), positions 1582 to 1602; antisense—CCGTATGCTTGTCACACAT (adopted from reference 25, primer PE32), positions 2002 to 2020; genebank accession no. L20965). (2) PDE4B2: sense—TGCTATGGACAGCCTGCAG, position 341 to 359; antisense—TGTGAGAATATCCAGCC (adopted from reference 26; within the PDE4B subtypes, the primer detects only PDE4B2), positions 830 to 846; genebank accession no. M97515). (3) PDE4C: sense—ATGAGGAGGAAGAAGAG-GAGGGG, position 2117 to 2139; antisense—AGTCCTCTG-GTTGTCGAGG, position 2229 to 2247; genebank accession no. Z46632. (4) PDE4D: sense—GCAAGATCGAGCACCT-AGCA (adopted from reference 25), positions 467-480; antisense—ACCAGACAACCTCTGCTATTC (adopted from reference 25), positions 956-971; genebank accession no. L20970. The amplimers used for IL-4 expression analysis were adopted from Jung et al (sense—AACACAACCTGAGAAGGAAAC-CTTC, positions 247 to 270; antisense—GCTCGAACACTTT-GAATATTTCTC, positions 499 to 522; gene bank accession no. M13982).²⁷

The amplimers for the β2m gene were described previously.²³ In order to determine the appropriate number of cycles for a semiquantitative estimation of the PCR products, the exponential range (number of PCR cycles versus signal intensity) of PCR under the chosen conditions was determined for each pair of amplimers. Accordingly, for the semiquantitative cDNA-PCR analysis the following number of cycles were used: 30 cycles (PDE4A), 28 cycles (PDE4B2), 28 cycles (PDE4C), 28 cycles (PDE4D), 28 cycles (IL-4), and 19 cycles (β2m). The amplimers used for analysis of PDE4A expression also detect the PDE4C gene that can be differentiated with restriction enzyme digest analysis according to Engels et al.²⁵

Determination of tumor necrosis factor-α release

Monocytes were incubated in 96 well plates at a density of 5×10^4 cells per 200 μl RPMI 1640 medium containing 10% heat-inactivated human AB serum with or without lipopolysaccharide (LPS) (1 ng/ml; *Salmonella abortus equi* endotoxin; Sigma, Deisenhofen, Germany) at 37°C in an incubator at 5% CO₂. PDE inhibitors or vehicle control (0.1% dimethylsulfoxide, final concentration) were added 30 minutes before LPS stimulation. 13 hours later, supernatants were removed and frozen at -80°C until further use. Total TNF concentration was determined with a commercially available ELISA kit (Immunotech, Hamburg, Germany) according to the manufacturer's protocol.

PBMC proliferation assay

PBMC (2×10^5) were seeded in 96 well plates in 200 μl RPMI 1640 medium containing 10% heat-inactivated FCS and stimulated with 2.5 μg/ml PHA for the times indicated at 37°C in an incubator at 5% CO₂. Proliferation of T cells was

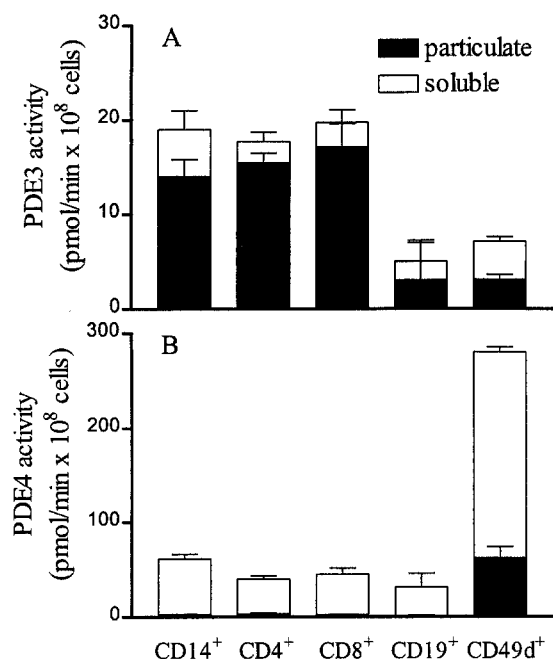


FIG. 2. PDE3 (A) and PDE4 (B) activity profiles of various leukocyte populations isolated from peripheral blood of normal donors determined at a substrate concentration of 0.5 $\mu\text{mol/L}$ cAMP. Data represent mean values $\pm$ SD of individual preparations: CD4⁺ and CD8⁺ T lymphocytes ($n = 3$); CD19⁺ B lymphocytes ($n = 5$); and CD14⁺ monocytes and CD49d⁺ eosinophils ($n = 6$). Note change in ordinate scale.

determined by incorporation of [³H] thymidine, which was present during the last 18 hours. PDE inhibitors were added 30 minutes before stimulation in a final dimethylsulfoxide concentration of 0.1%, and proliferation was determined at 72 hours.

Statistics

Data were analyzed with Student's *t* test when applicable or with Welch's approximate *t*-solution test when variances were nonhomogenous. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

PDE activity profiles of human blood leukocytes

We first determined the PDE activity profiles of human leukocytes from normal and atopic donors. The activities of PDE3 and PDE4, which are known to be the predominant isoenzymes in inflammatory cells, are shown in Fig. 2 for normal leukocytes. The majority of PDE3 activity was located in the particulate fraction. T lymphocytes and monocytes had threefold higher PDE3 activities compared to B cells and eosinophils. In general, PDE3 activity was low (20% or less of total cAMP hydrolyzing activity) compared to PDE4 activity, except for T cells (50% or less of total cAMP hydrolyzing activity). In lymphocytes and monocytes PDE4 activity was found exclusively in the soluble fraction, whereas in eosinophils about 20% of the rolipram-sensitive activity was membrane bound.

Neither PDE2 (cGMP-stimulated PDE) nor PDE5

TABLE I. Soluble PDE4 activities of human monocytes, lymphocytes, and eosinophils isolated from peripheral blood of normal and atopic individuals

	PDE4 activity (pmol/10 ⁸ cells $\times$ min)	
	Normal	Atopic
Monocytes	59 $\pm$ 5	65 $\pm$ 12
B lymphocytes	38 $\pm$ 9	33 $\pm$ 10
CD4 ⁺ T lymphocytes	29 $\pm$ 13	38 $\pm$ 11
Eosinophils*	218 $\pm$ 5	246 $\pm$ 14

Total cAMP PDE activities were measured at a substrate concentration of 0.5 $\mu\text{mol/L}$ cAMP. PDE4 activities represent the rolipram (10 $\mu\text{mol/L}$)-inhibited portion of total cAMP hydrolysis. Data are mean values $\pm$ SD from six individual determinations.

*Values taken from Tenor et al.⁴⁵

(cGMP-specific PDE) activity was found in leukocytes. PDE1 activity (Ca^{2+} /CaM-stimulated PDE) was measurable in some but not all T cells preparations and was absent in all other leukocytes (data not shown).

Surprisingly, PDE3 and PDE4 activities were identical in cells from normal and atopic donors (with mild or severe disease). No significant PDE changes (neither in total PDE activities, nor in cellular distribution of these enzymes) in monocytes, B cells, T cells, or eosinophils from atopic individuals were noted when compared to leukocytes from normal donors. Examples of soluble PDE4 activities from monocytes, B cells, CD4⁺ T cells, and eosinophils of the two groups of donors are shown in Table I.

mRNA expression profiles of PDE4 subtypes

In order to get a more detailed picture of the PDE4 subtypes present in various leukocyte subpopulations, we determined relative PDE4 mRNA expression profiles of normal and atopic leukocytes. Atopic status was verified by 10-fold higher IL-4 mRNA expression in CD4⁺ T cells from atopic patients (Table II).

Whereas PDE4A and PDE4B were expressed in all cell types investigated, no PDE4C was detectable in human leukocytes. Interestingly, high expression levels of PDE4D were observed in eosinophils and B cells, a weak PDE4D mRNA signal was detected in monocytes, and T cells either lacked significant expression of this PDE4 subtype (CD4⁺ cells) or expressed lower amounts (CD8⁺ cells) compared to B lymphocytes.

Again, no differences between normal and atopic cells (from patients with either mild or severe disease) were found, except increased PDE4A and PDE4B2 mRNA expression in CD4⁺ T cells from atopic patients (see Table II), which reflected in a slightly higher, but statistically not significant, mean PDE4 activity (see Table I).

Inhibition constants of PDE4-selective compounds

We next determined whether PDE4 activity from atopic cells would be differentially affected by PDE4-

TABLE II. PDE4 and IL-4 mRNA expression of inflammatory cells isolated from peripheral blood of normal and atopic subjects

	Donor	n	mRNA expression			
			PDE4A	PDE4B2	PDE4D	IL-4
Monocytes	Normal	3	++	+++	+	n.d.
	Atopic	4	++	+++	+	
CD4 ⁺ T cells	Normal	6	5.9 ± 8.1*	50.5 ± 16.3	(+)	9 ± 10
	Atopic	4	22.1 ± 7.6†	95.3 ± 8.1†	(+)	97 ± 25†
CD8 ⁺ T cells	Normal	3	++	+++	++	n.d.
	Atopic	3	+++	+++	++	
B cells	Normal	4	++	+++	+++	n.d.
	Atopic	3	++	+++	+++	
Eosinophils	Normal	6	+++	++	+++	n.d.
	Atopic	10	+++	++	+++	

Semiquantitative PCR analyses were performed as described in Materials and Methods. Because of the semiquantitative PCR, expression levels can be compared only vertically, not horizontally.

n, number of individual samples analyzed; (+), no significant signal; +, significant signal; ++, strong signal; +++, very strong signal; n.d., not determined.

*PDE4A, PDE4B2, and IL-4 data (mean ± SD) from CD4⁺ T cells are given as relative units (%) compared to the β2m signal, which is set at 100%. †p < 0.05 versus normal control.

selective inhibitors, as has been described by others.^{10, 28} Therefore, IC₄₀ values for the inhibition of PDE4 activity in the homogenate of monocytes (80% PDE4 and 20% PDE3 of total cAMP hydrolyzing activity; see Fig. 2) were determined for rolipram and Ro 20-1724. As depicted in Table III, identical IC₄₀ values for both PDE4 inhibitors were obtained in both atopic and normal individuals.

Because we failed to detect any PDE4 abnormalities in the basal state in purified leukocytes from atopic patients that is, after isolation, we next determined whether any functional differences were present related to PDE variations under stimulated conditions.

Suppression of LPS-induced TNF release from monocytes by PDE4 inhibitors

Similar quantities of TNF were released in both groups of monocytes exposed to LPS (1 ng/ml) for 13 hours (3641 ± 2422 pg/ml in normal monocytes [n = 3]; 3805 ± 1329 pg/ml in monocytes from atopic donors [n = 3]). Moreover, LPS-induced TNF release was identically suppressed in normal and atopic monocytes by rolipram, Ro 20-1724, or RS-25334²⁹ (Fig. 3), which is in accordance with the identical IC₄₀ values found for PDE4 inhibitors in the monocyte lysates (see Table III).

Suppression of polyclonal T cell proliferation by PDE inhibitors

Our final focus was on a functional assay in a less purified cellular system, that is, on PHA-induced T lymphocyte proliferation in PBMC and its modulation by PDE inhibitors, because a diminished proliferative capacity^{7, 8, 9} and a higher susceptibility of atopic PBMCs to PDE inhibitors have been described in this experimental setting.¹⁰

Fig. 4, A, shows the kinetics of DNA synthesis as a proliferative marker of PBMCs stimulated by the T cell

TABLE III. Inhibition of PDE4 activity in the homogenate of normal and atopic human monocytes

PDE4 inhibitor	IC ₄₀ (μmol/L)	
	Normal monocytes	Atopic monocytes
Rolipram	0.17	0.14
Ro 20-1724	0.59	0.44

Concentration-dependent inhibition curves were obtained on the homogenate of monocytes. PDE4 inhibitors were used at concentrations ranging from 0.01 μmol/L to 10 μmol/L (rolipram) and from 0.1 μmol/L to 100 μmol/L (Ro 20-1724). Because 20% of the total cAMP hydrolyzing activity of monocytes is attributable to PDE3 (see Fig. 2), IC₄₀ values for the PDE4 inhibitors are given. Mean values from three independent experiments are listed.

mitogen PHA for 3 days. The proliferative response of cells isolated from blood of atopic donors was identical to the response of normal cells at any time point investigated as was the concentration-dependent inhibition of proliferation observed for the PDE4-selective inhibitor rolipram (see Fig. 4, B) and for zardaverine, a mixed PDE3/4 inhibitor (see Fig. 4, C). Rolipram at 10 μmol/L inhibited the proliferative response of PBMC from normal and atopic donors by 46 ± 15% and 40 ± 20%, respectively (see Fig. 4, B). Zardaverine was more effective in inhibiting proliferation (68 ± 7% in normal cells [p < 0.01 versus rolipram] and 67 ± 9% in atopic PBMC [p < 0.01 versus rolipram] at 10 μmol/L, respectively) (see Fig. 4, C).

DISCUSSION

The present study was carried out to compare the PDE profiles of human leukocytes from normal and atopic donors. The use of optimized purification protocols provided the basis for a comprehensive, detailed PDE analysis in each individual cell type of interest. This

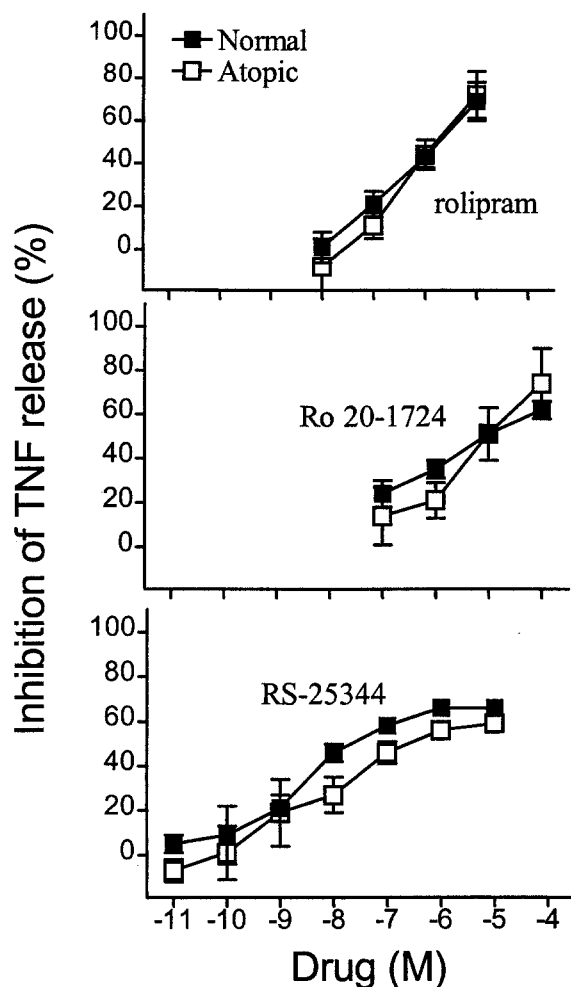


FIG. 3. Suppression of LPS-induced TNF release from CD14⁺ human monocytes by PDE4-selective inhibitors. PDE inhibitors or solvent (0.1% dimethylsulfoxide, final concentration) were added 30 minutes before LPS (1 ng/ml). 13 hours after the start of incubation, supernatants were collected and frozen at -80°C until TNF determinations were performed by ELISA. Data are mean values $\pm$ SD of three independent experiments performed in duplicate.

is particularly important, because PDEs are ubiquitously expressed enzymes and, therefore, contaminating platelets and other leukocyte subpopulations could influence the PDE analysis of a given population, which in turn would render proper data interpretation more difficult.

The prominent role of PDE4 in inflammatory human blood cells was confirmed in our study, and the mRNA expression patterns of the PDE4 subtypes showed hardly any qualitative differences among the various leukocyte populations. As previously reported^{25, 30, 31} PDE4A, PDE4B (splice variant PDE4B2), and PDE4D were present, whereas PDE4C was lacking in all cell types investigated.

Increased PDE4 activities in leukocytes from atopic subjects have been described in the past. Table IV summarizes present knowledge pertaining to atopy and PDE alterations that has been addressed in the scientific

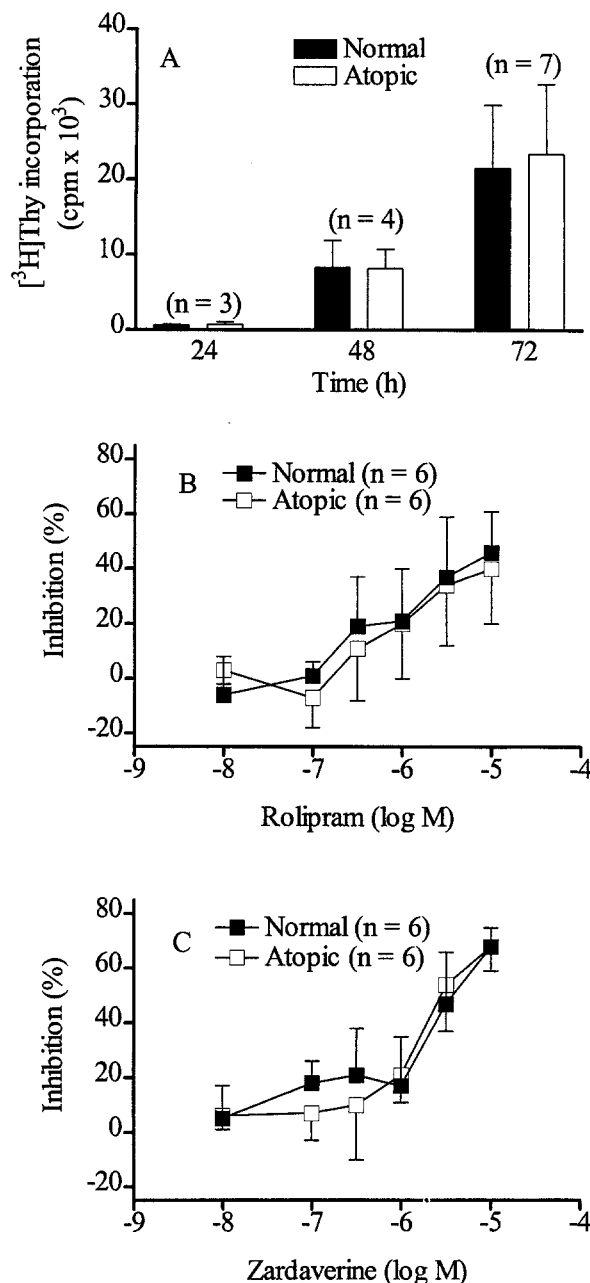


FIG. 4. Proliferation of polyclonally stimulated (with PHA, 2.5 $\mu\text{g/ml}$) PBMC (2×10^5 per well) isolated from peripheral blood of normal and atopic individuals. PDE inhibitors or solvent (0.1% dimethylsulfoxide, final concentration) were added 30 minutes before PHA: **A**, Time course of proliferative response. **B**, Concentration-dependent inhibition of T cell proliferation by the PDE4-selective inhibitor rolipram. **C**, Concentration-dependent inhibition of T cell proliferation by the mixed PDE3/4 inhibitor zardaverine. *n*, number of independent experiments performed.

literature and that creates a basis for the following discussion.

Surprisingly, we failed to detect increased PDE4 activity in leukocytes from atopic donors, which has been initially described in PBMC fractions of atopic donors⁴; the critical source of this higher, atopy-related PDE4

TABLE IV. Atopy and PDE alterations: controversial findings

Parameter investigated	Finding
PDE activity	• Increased in atopy (refs. 4, 5, 28)
PDE inhibition	• No significant alteration (refs. 22, 44, and 45 and this study) • Lower IC₅₀ values of PDE inhibitors in atopic cells (refs. 28, 36, 46) • Identical IC₅₀ values in atopic and normal cells (refs. 36 and 45 and this study)
T cell proliferation	• Diminished in atopy (refs. 7-9) • Identical in normal and atopic cells (refs. 10, 35, 37, and this study)
Inhibition of T cell proliferation	• Higher sensitivity of atopic cells (ref. 10) • Identical sensitivity of atopic and normal cells to PDE inhibitors (ref. 35 and this study)

activity is the monocyte.⁵ In atopic monocytes purified according to our experimental protocol, we found no evidence for imbalances in mRNA transcripts for the PDE4 subtypes (see Table II), for increased PDE4 activity⁵ (data not shown; see Table I), for higher susceptibility to Ro 20-1724²⁸ (see Table III), or for Ca²⁺/CaM dependency⁶ (not shown) of the enzyme. In addition, the lack of any differences in LPS-induced TNF production and its inhibition by PDE4 selective compounds (see Fig. III; reviewed in reference 2) between monocytes from the two different donor groups are in line with these observations.

Differences in methodology may provide a possible explanation for the Ca²⁺/CaM-stimulated PDE activity that was found by Chan et al.⁶ but not by us. It has been shown that the monocytic fraction of peripheral blood leukocytes consists of monocytes at various stages of differentiation including a CD14/CD16 double-positive subpopulation (CD14⁺/CD16⁺) that is prominent in blood from patients with asthma.³² These CD14⁺/CD16⁺ cells express a macrophage-like phenotype.³³ Because human macrophages, in contrast to normal peripheral blood monocytes (CD14⁺/CD16⁻), contain high Ca²⁺/CaM-dependent PDE1 activity,^{22, 34} it is likely that this PDE1 activity is detectable in monocytes, purified by plastic adherence, from asthmatic or atopic subjects.⁶ Because we used anti-CD16 antibodies to deplete granulocytes and NK cells, we also depleted CD14⁺/CD16⁺ monocytes and possibly lost PDE1 activity as a consequence of this isolation technique for monocytes. It remains to be clarified whether the absence of this CD14⁺/CD16⁺ monocyte subpopulation could also explain the lack of increased PDE4 activity or diminished PDE4 inhibitor sensitivity.

The only hint of altered PDE regulation in our study was the increased mRNA expression of PDE4A and PDE4B2 found in atopic T cells (see Table II) and, to a lesser extent, in atopic PBMC (data not shown). However, overall PDE4 activities were not significantly affected by the increased mRNA expression of these two PDE4 subtypes, although a tendency to an increased mean PDE4 activity was noted in atopic T lymphocytes

(see Table I). This lack of difference in T lymphocyte PDE4 activity is further supported by the data obtained in a functional T cell assay, namely, PHA-induced proliferation of PBMC. The identical PDE4 inhibitor sensitivity of proliferating T cells found under our conditions differs from the results published by Banner et al.,¹⁰ but fit those reported by Essayan et al.³⁵ and the findings of Chan et al., who described identical inhibition values of PDE4 activity by Ro 20-1724 in normal and atopic T lymphocyte homogenates.³⁶ Moreover, identical proliferative capacity in normal and atopic PBMC has been mentioned in at least three independent publications,^{10, 35, 37} which is in agreement with our results (see Fig. 4, A). In T lymphocytes, both PDE4 and PDE3 seem to play a functional role, because the mixed PDE3/4 inhibitor zardaverine was more effective compared to rolipram, a selective PDE4 inhibitor, in inhibiting the proliferative response of PBMC after polyclonal T cell stimulation. This finding is in accordance with data previously published.^{10, 35-39}

It is of note that the proliferative response to PHA and the susceptibility to PDE inhibitors were unaffected by indomethacin (10 μ mol/L), a compound used to block any endogenous synthesis of cyclooxygenase-derived arachidonic acid metabolites. In particular, higher amounts of prostaglandin E₂ are produced by atopic monocytes under basal conditions^{40, 41} and, by stimulating cAMP generation, could act in synergy with PDE inhibitors. Our results, however, do not support the assumption that altered prostaglandin E₂ production in atopy is involved in the regulation of T cell proliferation.

Overall, our data are in accordance with the PDE profile found in human CD4⁺ and CD8⁺ T cells^{39, 42} and underline the functional role of both PDE3 and PDE4 in controlling T cell growth.^{10, 35-39, 43}

In summary, the data obtained with our methods provide no convincing evidence for increased PDE4 activity in atopic patients, and thus the present study differs from several reports on PDE4 dysregulation in atopy.⁴¹ As already discussed above, differences in the purification protocol for the individual cell types may be substantially influencing the phenotype composition of

such preparations. However, several other explanations for the discrepancies have to be considered. First, the selection of the patients (disease status, medication) may be critical. Over the past few years, however, we have investigated cells from patients with mild or severe atopy and have never found any differences compared to normal controls. Moreover, patients investigated by Hanifin and collaborators seem to belong to the group with severe atopy, whereas Banner et al. used blood from mildly atopic patients for their PBMC studies.¹⁰ These facts imply that selection of patients may not be particularly critical to the outcome of such studies, but this issue cannot be ruled out completely.

Second, the relatively long isolation procedure that has been used in our studies could result in a loss of differences in PDE protein expression or activity. The fact that atopic PBMC fractions, which can be obtained 1 to 2 hours after blood collection, were equally sensitive compared to normal cells with regard to functional inhibition by PDE inhibitors (see Fig. 4) argues against the duration of experimental procedures as being a major cause for the lack of PDE4 imbalances observed in this study. Although atopic PBMC showed increased PDE4 mRNA expression (PDE4B2, not shown), we refrained from PDE activity measurements in the PMBC fraction because cellular heterogeneity and platelet contamination make data interpretation difficult, as discussed at the beginning.

Third, different kinetic properties such as a higher K_m value for cAMP of an "atopy-related PDE4" could account for the disparate results published by different investigators. However, in the presence of a 10-fold higher substrate concentration (namely, 5 μ mol/L cAMP), PDE4 activities of atopic lymphocytes were indistinguishable from those of normal controls (data not shown).

Fourth, it could be speculated that the changes in PDE4 activity of atopic cells in vivo are regulated completely in the short-term or are dependent on the continuous presence or absence of appropriate modulators. Such (extracellular) factors would be lost immediately after blood collection and, therefore, existing differences would not be found.

In conclusion, despite some tendencies in T cells, an altered PDE activity profile or a higher sensitivity toward isoenzyme-selective PDE inhibitors in atopic leukocytes was not found when compared to normal controls under our experimental conditions. The pathophysiologic significance of the relative increase of the PDE4A and PDE4B2 mRNA levels in T cells is unclear. It remains to be clarified which factors (e.g., disease state, criteria for selection of the patients, experimental conditions) may cause the contradictory outcome of such in vitro investigations. In our opinion, the question of a putative "atopy-specific" PDE remains controversial and still awaits final elucidation.

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