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Phosphodiesterase profile of human B lymphocytes from normal and atopic donors and the effects of PDE inhibition on B cell proliferation

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1 $CD19^+$ B lymphocytes were purified from the peripheral blood of normal and atopic subjects to analyse and compare the phosphodiesterase (PDE) activity profile, PDE mRNA expression and the importance of PDE activity for the regulation of B cell function.

2 The majority of cyclic AMP hydrolyzing activity of human B cells was cytosolic PDE4, followed by cytosolic PDE7-like activity; marginal PDE3 activity was found only in the particulate B cell fraction. PDE1, PDE2 and PDE5 activities were not detected.

3 By cDNA-PCR analysis mRNA of the PDE4 subtypes A, B (splice variant PDE4B2) and D were detected. In addition, a weak signal for PDE3A was found.

4 No differences in PDE activities or mRNA expression of PDE subtypes were found in B cells from either normal or atopic subjects.

5 Stimulation of B lymphocytes with the polyclonal stimulus lipopolysaccharide (LPS) induced a proliferative response in a time- and concentration-dependent manner, which was increased in the presence of interleukin-4 (IL-4). PDE4 inhibitors (rolipram, piclamilast) led to an increase in the cellular cyclic AMP concentration and to an augmentation of proliferation, whereas a PDE3 inhibitor (motapizone) was ineffective, which is in accordance with the PDE profile found. The proliferation enhancing effect of the PDE4 inhibitors was partly mimicked by the cyclic AMP analogues dibutyryl (db) cyclic AMP and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate, Sp-isomer (dcl-cBIMPS), respectively. However, at concentrations exceeding 100 μM db-cyclic AMP suppressed B lymphocyte proliferation, probably as a result of cytotoxicity. Prostaglandin E_2 (PGE_2, 1 μM) and forskolin (10 μM) did not affect B cell proliferation, even when given in combination with rolipram.

6 Inhibition of protein kinase A (PKA) by differentially acting selective inhibitors (KT 5720, Rp-8-Br-cyclic AMPS) decreased the proliferative response of control cells and reversed the proliferation enhancing effects of rolipram.

7 Importantly, PDE4 activity in LPS/IL-4-activated B lymphocytes decreased by about 50% compared to unstimulated control values.

8 We conclude that an increase in cyclic AMP, mediated by down-regulation of PDE4 activity, is involved in the stimulation of B cell proliferation in response to LPS/IL-4. B cell proliferation in response to a mitogenic stimulus can be further enhanced by pharmacological elevation of cyclic AMP.

Keywords: $CD19^+$ B cells; atopic dermatitis; PDE4 subtypes; rolipram; cyclic AMP; PDE isoenzymes; protein kinase A; PKA inhibitors

Introduction

Among the large variety of cellular responses that are orchestrated by the adenosine 3′:5′-cyclic monophosphate (cyclic AMP)/protein kinase A (PKA) pathway, the anti-inflammatory properties of this second messenger system have attracted particular interest during recent years. Cyclic AMP is synthesized through the action of adenylyl cyclase and its sole route of degradation is by the action of cyclic AMP phosphodiesterases (PDE), a multigene family (for recent reviews see Beavo *et al*., 1994; Bolger, 1994; Beavo, 1995; Loughney & Ferguson, 1996). In inflammatory cells, members of the low K_m cyclic AMP-specific PDE4 are the predominant isoenzymes in terms of expression and distribution. PDE4 is believed to play an important immunoregulatory role, since many inflammatory reactions are suppressed by PDE4-selective inhibitors such as rolipram, which is in accordance with the long-known anti-inflammatory properties of cyclic AMP elevating agents and cyclic AMP itself. In monocytes, the production of proinflammatory mediators is drastically decreased (Semmler *et al*., 1993; Prabhakar *et al*., 1994; Seldon *et al*., 1995). Similarly, various functions of other leucocytes including eosinophils (Dent *et al*, 1994; Hatzelmann *et al*., 1995; Souness *et al*., 1995; Tenor *et al*, 1996), neutrophils (Wright *et al*., 1990; Schudt *et al*., 1991; Fonteh *et al*., 1993), macrophages (Schade & Schudt, 1993; Fischer *et al*., 1993; Gantner *et al*., 1997a), basophils (Peachell *et al*., 1992), mast cells (Torphy *et al*., 1992) and T lymphocytes (Robicsek *et al*., 1991; Essayan *et al*., 1994; Crocker *et al*., 1996; Gantner *et al*., 1995; Giembycz *et al*., 1996) are strongly impaired. B cells as well as eosinophils even undergo apoptosis following cyclic AMP elevation/PDE inhibition (Lømo *et al*., 1995; Hallsworth *et al*., 1996).

In view of these anti-inflammatory properties of cyclic AMP, it is not surprising that there is considerable interest in

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the development of PDE4 inhibitors for the treatment of chronic inflammatory diseases such as asthma and atopic dermatitis, where all of the inflammatory cell populations mentioned above play a critical role. Moreover, it has been found that in leucocytes of atopic patients a higher PDE4 activity exists (Grewe *et al*., 1982; Chan *et al*., 1993) and that peripheral blood mononuclear cells (PBMC) from such patients are more sensitive to PDE inhibitors (Chan & Hanifin, 1993; Banner *et al*, 1995). This provides a further rationale for the development of PDE inhibitors as anti-inflammatory drugs.

However, many studies on PDE isoenzyme distribution and functional consequences of PDE inhibition in leucocytes have been performed in mixed cell populations such as PBMC. This prompted us to analyse individually, and to characterize functionally highly purified populations of human freshly isolated inflammatory cells relevant in asthmatic and atopic disorders. Analysis of monocytes and macrophages, neutrophils, eosinophils and $CD4^+$ as well as $CD8^+$ T lymphocytes confirmed the prominent role of PDE4 isozymes for the regulation of inflammatory responses in these cells (for reviews see Tenor & Schudt, 1996; Dent & Giembycz, 1996).

In B cells, both stimulating and inhibitory effects have been attributed to cyclic AMP elevation (Holte *et al*., 1988; Roper & Phipps, 1992; Garrone & Banchereau, 1993; Coqueret *et al*., 1996). Whereas β-adrenoceptor agonists, adenylyl cyclase activators, prostaglandin E_2 (PGE_2) and cyclic AMP analogues have been studied with regard to B cell proliferation and function, the effects of PDE isoenzyme-selective inhibitors have not yet been determined. For human $CD19^+$ B lymphocytes, details of the PDE isoenzyme profile and the functional consequences of PDE inhibition are not known. Thus, in the present study we used highly purified B lymphocytes from both healthy donors and atopic patients and compared their PDE mRNA expression and activity profile under control and stimulated conditions. Furthermore, the influence of selective PDE inhibitors on polyclonal B cell proliferation was studied.

Methods

Subjects

Normal healthy donors who had no history of asthma, allergic rhinitis or atopic dermatitis were selected for this study. Additionally, the blood of a separate group of patients suffering from atopic dermatitis was used. The patients were off medication (corticosteroids) for at least 3 weeks before the investigation. This study was approved by the local ethics committee and carried out according to national guidelines.

Cell preparation

For each experiment 250 ml of peripheral venous blood was drawn from a single volunteer. Citrate (0.31%) was used as an anti-coagulant. The blood was diluted 1.6 fold with PBS (pH 7.4) before centrifugation at $220 \times g$ at 20°C for 20 min. The cell pellet was layered on a Percoll gradient (p 1.077 g ml^{-1}) and the interphase containing the peripheral blood mononuclear cells (PBMC) was obtained following centrifugation at $800 \times g$. Cells were washed twice in elutriation medium (PBS, 2% heat-inactivated human AB serum, 2 mM EDTA, 5 mM glucose, pH 7.4) before countercurrent centrifugal elutriation of the cells with a J2-MC centrifuge equipped with a JE-6B rotor (Beckman, U.S.A.). The lymphocyte containing fraction (platelet-free, >95% purity) was obtained at a flow rate of 29 ml min^{-1} and a rotor speed of 3000 r.p.m. Pure B lymphocytes were obtained by positive selection using magnetic-beads-conjugated anti-CD19 Ab and detaching Ab according to the manufacturer's protocol (Dynal, Oslo, Norway). B cell purity ($CD19^+$ cells) was checked by flow cytometry (Coulter, Hamburg, Germany) and was always >99%. No $CD3^+$ T cells or $CD14^+$ monocytes were detectable which is in accordance to the unresponsiveness of these cells towards the T cell mitogen, phytohaemagglutinin (PHA, data not shown).

Preparation of subcellular B lymphocyte fractions

Freshly prepared $CD19^+$ B cells were resuspended at 10^7 cells ml^{-1} in homogenization buffer (10 mM HEPES, pH 8.2, 1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EGTA, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 5 μM pepstatin A, 10 μM leupeptin, 50 μM PMSF, 10 μM soybean trypsin inhibitor and 2 mM benzamidine) and disrupted by sonication (Branson 250 sonifier, output control 10%, duty cycle 1). The complete disruption of the cells (>98%) was checked via trypan blue exclusion or, alternatively, via lactate dehydrogenase (LDH) release. The homogenate was spun at $1000 \times g$ for 5 min to remove viable cells (1–2%) and nuclei. The samples were then centrifuged at $100{,}000 \times g$ for 60 min at 4°C. The supernatant was decanted and the pellet was resuspended in the corresponding volume of homogenization buffer. These two fractions (further referred to as the soluble and particulate fraction, respectively) were stored at −80°C until further use.

PDE activity assay

PDE activity was determined as described by Thompson *et al*. (1979) with some modifications (Bauer & Schwabe, 1980). In brief, the enzyme containing fractions were assayed in a final volume of 200 μl containing 60 mM Tris HCl, pH 7.4, 5 mM $MgCl_2$, 0.5 μM cyclic AMP or cyclic GMP (28 000 c.p.m. [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP) and were incubated in the presence or absence of activators or inhibitors for 30 min at 37°C. The reaction was terminated by the addition of 50 μl 0.2 N HCl and the assay mixture was left on ice for a further 15 min. *Crotalus atrox* snake venom (0.5 mg ml^{-1}) was added for 15 min at 37°C and the assay mixture was then loaded onto QAE-Sephadex A-25 columns (1 ml bed volume) and eluted with 2 ml ammonium formiate (30 mM, pH 6.0). The radioactivity in the eluate was counted in a liquid scintillation counter (Beckman). PDE isoenzyme activity calculations in the soluble as well as in the particulate B cell fractions were performed, making use of activators and the PDE isoenzyme-selective inhibitors motapizone (PDE3), rolipram (PDE4) and zaprinast (PDE5), as described by Tenor *et al*. (1995a). PDE7 activity was defined as the residual cyclic AMP hydrolyzing activity in the presence of motapizone (1 μM) and rolipram (10 μM), respectively.

PDE expression analysis by cDNA-PCR

Preparation of RNA, conditions for cDNA-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 (Wilisch *et al*., 1993; Beck *et al*., 1995). The buffer for PCR provided by the supplier (Amersham) was applied as recommended. After the staining of the gels with ethidium

bromide, the signals were digitized by the CS-1 videoimager (Cybertech, Berlin, Germany). Signal intensities calculated with the WINCAM densitometric software (Cybertech) assigned to the various genes of interest were referred to those corresponding to the β2-microglobulin (β2m) gene obtained by a simultaneously performed PCR with an aliquot of the same cDNA.

The following amplimers for detecting PDE mRNA expression were used: PDE3A (sense: CAACTCCTATGATT-CAGCA, position 3061 to 3079; antisense: CTGGTCTGGCTTTTGGGTT, position 3427 to 3445; genebank accession# M91667); PDE3B (sense: TCTATATCTTTCTCGCCCAG, position 2580 to 2598; antisense: CTTCTTCATCTCCCTGCTC, position 2881 to 2899, genebank accession# U38178); PDE4A (sense: TCAGAGCTGGCGCTTATGTAC (adopted from Engels *et al*., 1994, primer PE21), position 1582 to 1602; antisense: CCGTATGCTTGTCACA CAT (adopted from Engels *et al*., 1994, primer PE32), position 2002 to 2020; genebank accession# L20965); PDE4B2 (sense: TGCTATGGACAGCCTGCAG; position 341 to 359; antisense: TGTGAGAATATCCAGCC (adopted from Obernolte *et al*., 1993), position 830 to 846, genebank accession# M97515), PDE4C (sense: ATGAGGAGGAAGAAGAGGAGGGG; position 2117 to 2139; antisense: AGTCCTCTGGTTGTCGAGG; position 2229 to 2247, genebank accession# Z46632); PDE4D (sense: GCAAGATCGAGCACCTAGCA; position 461 to 480; antisense: ACCAGACAACTCTGCTATTC; position 952 to 971, genebank accession# L20970, according to Engels *et al*., 1994); PDE7 (sense: GTCTAGTAAGCTTAA; position 557 to 571; antisense: GGCTTATTCTCACATCTG; position 939 to 956, genebank accession# L12052, according to Michaeli *et al*., 1993).

With the exception of the PDE4B primer, which detects the short form PDE4B2 variant only, the PDE primers we used were generic, i.e. they amplify all splice variants of a certain gene. The amplimers for the β2m gene were adopted from a previous study (Wilisch *et al*., 1993). 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 polymerase chain reaction under the chosen conditions was determined for each pair of amplimers. Accordingly, for the semiquantitive cDNA-PCR analysis the following number of cycles were used: 26 cycles (PDE3A), 28 cycles (PDE3B), 30 cycles (PDE4A), 28 cycles (PDE4B2), 28 cycles (PDE4C), 28 cycles (PDE4D), 28 cycles (PDE7), 19 cycles (β2m). The amplimers used for analysis of PDE4A expression also detect the PDE4C gene, which can be differentiated with restriction enzyme digest analysis according to Engels *et al*. (1994).

Cyclic AMP measurements

Intracellular cyclic AMP content was determined by an enzyme immunoassay (Biotrak EIA) according to the non-acetylation assay instructions provided by the supplier (Amersham Life Sciences, Braunschweig, Germany) with the modification of adding the unselective PDE inhibitor isobutyl methyl xanthine (IBMX; 0.5 mM) to the extraction buffer to ensure inhibition of cyclic nucleotide breakdown by PDE activity.

B cell proliferation

B cells (2×10^5 per well containing 200 μl RPMI 1640 medium) were seeded in 96-well flat bottom plates in the presence of test compounds or the corresponding volume of vehicle (dimethylsulphoxide; DMSO, 0.1% final concentration). Thirty minutes later, cells were stimulated by the indicated concentrations of lipopolysaccharide (LPS) and interleukin-4 (IL-4) for 5 days. Proliferation was evaluated by standard liquid scintillation counting based on [^{3}H]-thymidine uptake which was present for the last 24 h of culture (0.2 μCi/well).

Cell culture media and reagents

RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% foetal calf serum (FCS), penicillin (50 iu ml^{-1}) and streptomycin (50 μg ml^{-1}) was used for all cultures. Purified *rhu*IL-4 was purchased from ICC Chemicals (Ismaning, Germany). Motapizone was kindly provided by Nattermann (Köln, Germany), and rolipram was obtained from Schering (Berlin, Germany). Rp-8-Br-cyclic AMPs and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′:5′-cyclic monophosphorothioate (dcl-cBIMPS) were from Biolog (Bremen, Germany), and KT 5720 was from Biomol (Hamburg, Germany). Lipopolysaccharide (LPS; *S. abortus equi*) and all reagents not further specified were purchased from Sigma (Deisenhofen, Germany).

Statistics

Unless otherwise stated, data are expressed as means $\pm$ s.d. of results carried out in at least three independent experiments with blood from different donors. Statistical significances were determined with unpaired Student's t test if applicable or according to Welch's approximate t solution if variances were nonhomogeneous by use of commercially available statistical programmes (GraphPad Software, San Diego, CA). $P<0.05$ was considered as statistically significant.

Results

PDE activity of resting human CD19$^+$ B lymphocytes from normal and atopic donors

An increased cyclic AMP hydrolyzing activity in leucocytes of atopic patients has been found previously (reviewed in Hanifin & Chan, 1995). However, in pure human B cells the PDE profile has not been analysed previously. This prompted us to determine PDE activity and PDE mRNA expression of B lymphocytes prepared from peripheral blood of normal and atopic individuals. Most of the cyclic AMP hydrolyzing activity of the soluble (cytosolic) fraction was inhibited by rolipram (10 μM) and therefore represents PDE4 activity. In addition, substantial PDE7 activity (residual activity in the presence of a PDE4 and a PDE3 inhibitor) was found, which was more prominent in the soluble compared to the particulate fraction. Overall, only marginal activity of PDE3 (motapizone-sensitive) was found, which was predominantly membrane-bound. Neither PDE1, PDE2 nor PDE5 activity were detectable in purified human B cells. The PDE activity profile was identical between normal (Figure 1a) and atopic individuals (Figure 1b).

To characterize PDE isoenzyme and subtype mRNA expression, we prepared mRNA from both normal and atopic B cells for cDNA-PCR analysis. As expected from the PDE activity profile, mRNA transcripts for PDE3, PDE4 and PDE7 were found (Figure 2). Among the known PDE3 subtypes, a weak PDE3A mRNA signal was noted, but no PDE3B mRNA was detectable. PDE4A, 4B2 and 4D mRNAs were present, but PDE4C was lacking in B cells. A very high mRNA signal was obtained for PDE7.

Again, no significant differences in PDE mRNA expression between B cells from healthy and atopic donors were noted (Figure 2). Since the PDE profile of B cells from atopic and healthy donors turned out to be identical, the further studies were carried out with cells from healthy volunteers.

Increased DNA synthesis following PDE4 inhibition in B cells stimulated by a combination of LPS and IL-4

To investigate functional consequences of PDE inhibition in human B cells, we tested the influence of PDE inhibitors on B cell proliferation. Based on the PDE isoenzyme pattern described previously, we concentrated on PDE4 and PDE3 selective compounds. Due to the lack of PDE7 selective inhibitors, the question of a possible regulatory role of this PDE family was not addressed.

In the first set of experiments, B cell proliferation was induced over various time periods (between 2 and 9 days) by polyclonal stimulation with combinations of LPS (0.1–10 μg ml^{-1}) and IL-4 (10–1000 u ml^{-1}). At day 5, stimulation with LPS (10 μg ml^{-1}) plus IL-4 (100 u ml^{-1}) was optimal with regard to [^{3}H]-thymidine incorporation (Figure 3), and therefore was used for the following experiments.

The prototype PDE4-selective inhibitor rolipram concentration-dependently increased the proliferative response of IL-4/LPS-stimulated CD19$^+$ B cells. A two fold higher thymidine incorporation rate was observed at concentrations $\geqslant 1$ μM rolipram (Figure 4). Analogous results were obtained with piclamilast, another PDE4-selective compound (210$\pm$23% proliferation at 1 μM, $n=4$). In contrast, the PDE3 inhibitor motapizone neither modulated B cell proliferation nor influenced the effect of the PDE4 inhibitor (Figure 4). Thus, PDE3 inhibition does not play a significant functional role in B cell proliferation, which is in agreement with the marginal PDE3 expression and activity present in these cells (see Figures 1 and 2).

Since PDE inhibitors are assumed to act via elevation of cyclic AMP, we measured the intracellular concentration of this second messenger. Indeed, a three fold increase in the cyclic

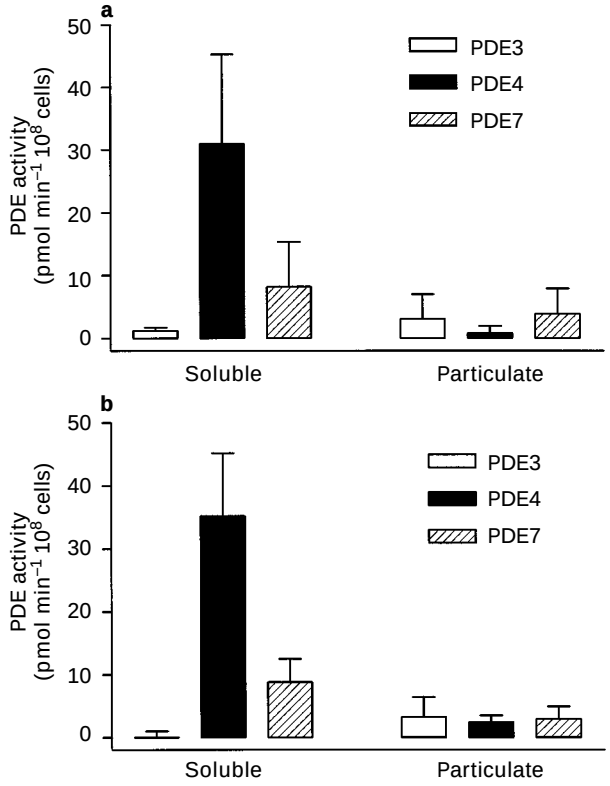

Figure 1 PDE activity profile of resting human B lymphocytes. PDE activities were determined in the soluble and particulate fraction of highly purified B cells isolated from normal (a) or atopic donors (b). Values were calculated on a per cell basis. Data representing PDE3 PDE4 and PDE7 are expressed as mean$\pm$s.d. from 5 independent preparations. No PDE1, PDE2 or PDE5 was detected.

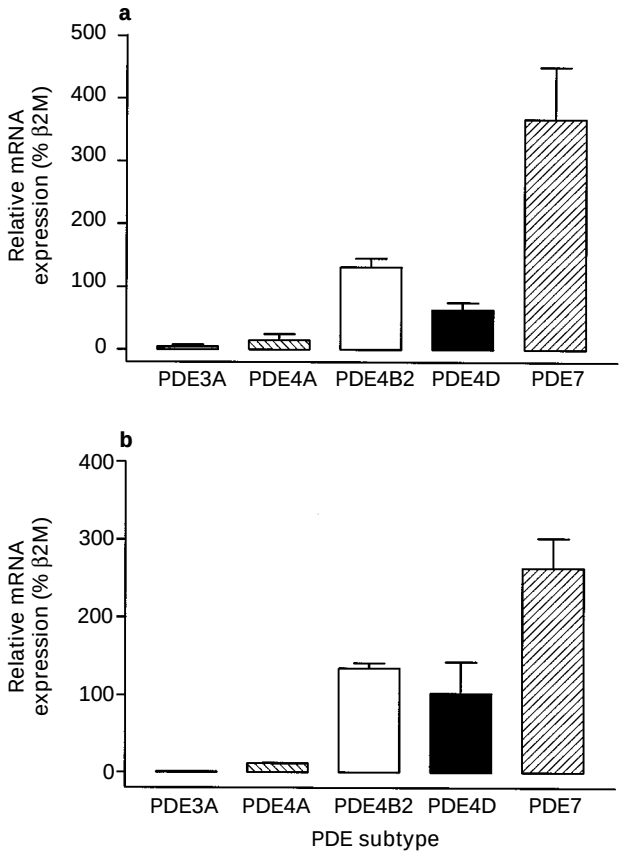

Figure 2 Relative PDE3, PDE4 and PDE7 subtype mRNA expression analysis by cDNA-PCR of resting human B cells from (a) normal or (b) atopic donors. Data are expressed as mean values$\pm$s.d. from 5–6 independent preparations.

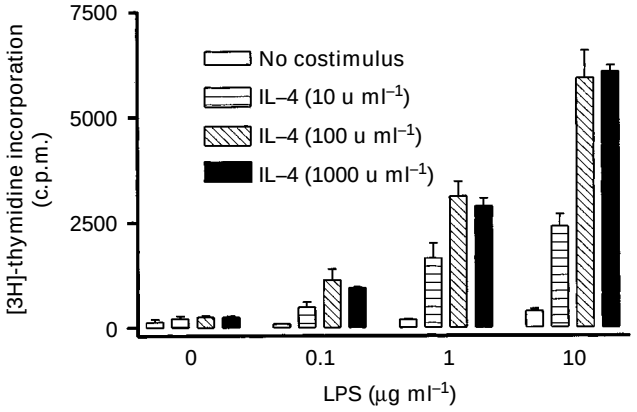

Figure 3 Polyclonal human B cell proliferation in response to LPS plus IL-4. B cells (2×10^5) were incubated in 200 μl culture medium with LPS in the absence or presence of various concentrations of IL-4. Four days later, [^{3}H]-thymidine was added (0.2 μCi per well) for another 24 h. Cells were then harvested and proliferation was determined by [^{3}H]-thymidine incorporation. Data represent mean values$\pm$s.d. of 4 independent experiments.

AMP levels was found 1 h after stimulation of the cells in the presence of rolipram compared to unstimulated controls or LPS/IL-4-treated cells (Table 1). We do not know whether, and, if so, to what extent and at what time point any significant changes in the cyclic AMP concentration were induced following LPS/IL-4 stimulation. Due to cell number limitations, the low cyclic AMP content of B cells, and the long experimental procedure we did not further expand these experiments.

We also examined the effect of the cell-permeable cyclic AMP analogues dibutyryl cyclic AMP (db-cyclic AMP) and dcl-cBIMPS on B cell proliferation. A bell-shaped concentration-response curve was obtained for db-cyclic AMP. At concentrations up to 100 μM both compounds mimicked the effect of the PDE4 inhibitors and increased IL-4/LPS-induced DNA synthesis rate by about 30% compared to control cells (Figure 4). However, at concentrations >300 μM, db-cyclic AMP strongly suppressed the proliferation of $CD19^+$ B cells (50–60% reduction, not shown) indicating that at least two cyclic AMP-dependent pathways might be involved in the regulation of DNA synthesis of human B cells. Trypan blue exclusion studies revealed evidence for the induction of cytotoxicity in the presence of 1 mM cyclic AMP after 5 days of LPS/IL-4 stimulation (68 ± 7% viable cells in the presence, 83 ± 5% viable cells in the absence of db-cyclic AMP; $n=5$, $P<0.05$), providing an explanation for the proliferation suppressing effects of high db-cyclic AMP concentrations.

In contrast to db-cyclic AMP and dcl-cBIMPS, both forskolin (0.01–10 μM), an adenylyl cyclase activator, and PGE_2 (0.001–1 μM) lacked efficacy (Figure 4). Moreover, both compounds failed to alter the concentration-response curve of rolipram at 10 μM (forskolin) or 1 μM (PGE_2), respectively (data not shown).

PKA activity is necessary for B cell proliferation

The influence of PKA inhibitors on LPS/IL-4-induced B lymphocyte proliferation in the absence or presence of rolipram was investigated. Remarkably, a dramatic inhibition of the proliferative response was observed under both experimental conditions. The two differentially acting PKA inhibitors tested (KT 5720, Rp-8-Br-cyclic AMPS) suppressed B cell proliferation in a concentration-dependent manner and reverted the proliferation-enhancing effect of rolipram (Figure 5).

PDE4 activity is decreased in proliferating B cells

Increased PDE4 activity, either due to induction of gene expression or posttranslational modification, has been

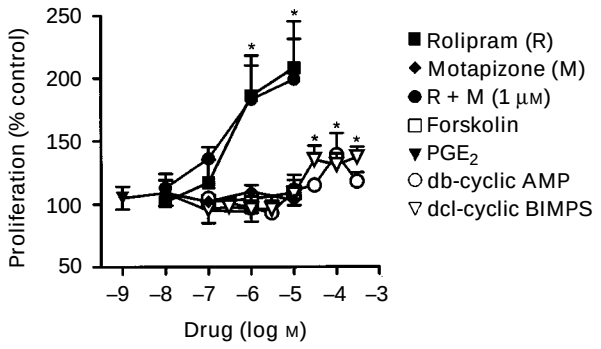

Figure 4 Influence of cyclic AMP elevating drugs on human B cell proliferation. B cells were plated on 96 well plates (5×10^5 cells in 200 μl) and incubated with test compounds or the corresponding volume of DMSO (0.1% final concentration) for 30 min. Proliferation was induced by LPS (10 μg ml^{-1}) plus IL-4 (100 u ml^{-1}) and determined at day 5 as described in legend to Figure 3. Values were calculated in relation to the proliferation signal of control incubations run in parallel (7550 ± 1480 c.p.m., set at 100%). Data are mean values from 6–14 independent experiments; vertical lines show s.d. *Indicates statistical significance with $P<0.01$ compared to control.

Table 1 Cyclic AMP elevation by rolipram following mitogenic stimulation of human B lymphocytes

Treatment	*Cyclic AMP* (fmol/10^6 cells)
None	1086 ± 612
LPS/IL-4	1037 ± 290
LPS/IL-4 + rolipram	3287 ± 339*

$CD19^+$ B cells were incubated for 1 h at 37°C in the presence of LPS (10 μg ml^{-1})/IL-4 (100 u ml^{-1}), LPS/IL-4 + rolipram (10 μM) or medium alone. Cells were washed twice, disrupted and cyclic AMP was extracted as described in the Methods section. Samples were stored at −20°C until cyclic AMP determination by EIA. Mean values ± s.d. from 4–5 independent experiments are given. *$P<0.05$ versus other groups.

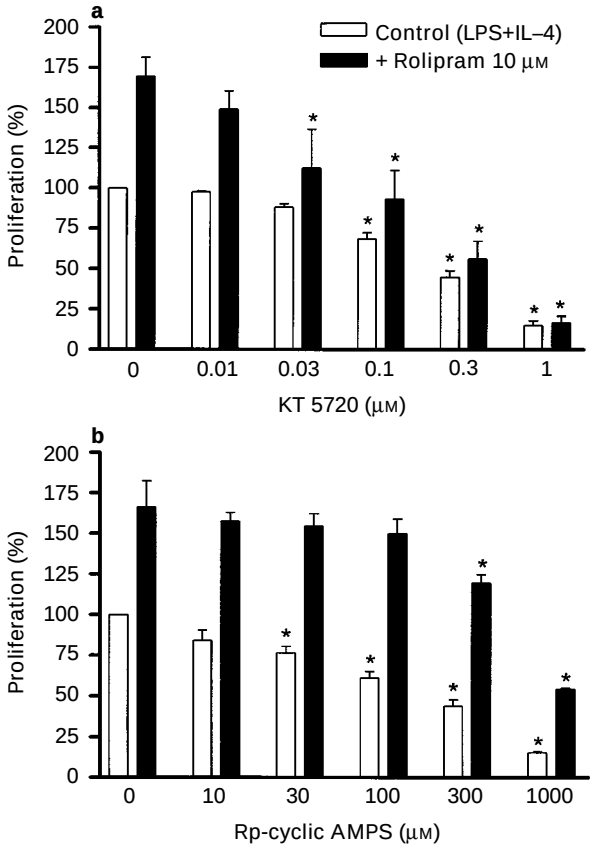

Figure 5 Influence of PKA inhibitors on human B cell proliferation. B cells were plated on 96 well plates (5×10^5 cells in 200 μl) and incubated with PKA inhibitors (a) KT 5720 or (b) 8-Br-Rp-cyclic AMPs or the corresponding volume of DMSO (0.1% final concentration) for 30 min. Proliferation was induced by LPS (10 μg ml^{-1}) plus IL-4 (100 u ml^{-1}) and determined at day 5 as described in legend to Figure 3. Values were calculated in relation to the proliferation signal of control incubations run in parallel (6850 ± 1210 c.p.m., set at 100%). Data are mean values ± s.d. from 4 (8-Br-Rp-cyclic AMPS) or 5 (KT 5720) independent experiments. *Indicates statistical significance with $P<0.01$ compared to the respective values in the absence of the PKA inhibitor.

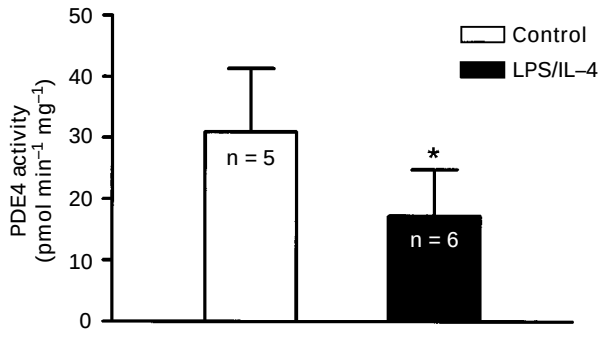

Figure 6 Decreased PDE4 activity in proliferating human B lymphocytes. B cells were either incubated with LPS (10 μg ml^{-1}) plus IL-4 (100 u ml^{-1}) or the same volume of medium as control. Five days later, cells were harvested and PDE4 activity was determined in the cytosolic fraction. Data are mean values $\pm$ s.d.; n = number of individual determinations. *Indicates statistical significance with $P<0.01$ compared to control.

repeatedly described in activated leucocytes (reviewed in Beavo, 1995). Thus, we wondered whether PDE4 activity would change upon induction of proliferation in human B lymphocytes. Five days following stimulation of CD19$^+$ cells by LPS (10 μg ml^{-1}) and IL-4 (100 u ml^{-1}), PDE4 activity was significantly decreased compared to unstimulated control cells kept in culture for the same time (Figure 6). Messenger RNA analyses of two independent B cell samples revealed evidence for a 60% downregulation of the main PDE4 subtype, i.e. PDE4B2, in proliferating B cells at day 5, suggesting that the reduced PDE4 activity is due to a reduced expression of the corresponding gene(s).

Discussion

The present study was conducted to identify the PDE enzymes that are involved in the regulation of cyclic AMP levels in human B cells. This was of particular interest, since a variety of B cell responses are modulated by cyclic AMP (Holte *et al*., 1988) and both stimulating and inhibiting effects (Simkin *et al*., 1987; Anastassiou *et al*., 1990; Roper & Phipps, 1992; Garrone & Banchereau, 1993; Coqueret *et al*., 1996) on activation parameters of B lymphocytes have been attributed to the cyclic AMP/PKA signalling cascade.

The isolation method we used provided B cells of high purity without contamination by platelets or other blood cells. Similar to other leucocyte populations from peripheral human blood (summarized in Tenor & Schudt, 1996), the vast majority of the cyclic AMP hydrolyzing PDE activity of B lymphocytes was identified as soluble PDE4, presumably composed of the subtypes PDE4A, B and D, respectively (see Figures 1 and 2) as indicated by the detection of the corresponding mRNA transcripts. In addition, a significant amount of PDE7 activity was measured. Only very weak PDE3 activity was found in B cells, which is surprising, since human T lymphocytes contain equally high activities of PDE4 and PDE3 (Tenor *et al*., 1995b; Giembycz *et al*., 1996), both of which are functionally involved in the inhibitory effects of cyclic AMP in this cell type (Robiczek *et al*., 1991; Essayan *et al*., 1994; Banner *et al*., 1995; Giembycz *et al*., 1996). Thus, the human peripheral blood lymphocyte PDE profile differs substantially between the T and B lineage.

A direct comparison of B cells from normal and atopic donors gave no evidence for an increased atopy-specific PDE4 activity, which is in agreement with recent PDE measurements performed in a variety of human leucocyte populations (Gantner *et al*, 1997b). According to Hanifin and coworkers atopic monocytes, but not atopic lymphocytes, contain increased PDE4 activity (reviewed in Hanifin & Chan, 1995), which is in accordance with our present data.

Our functional studies with PDE inhibitors on activated B cells reflect the PDE activity pattern present in these cells. Whereas PDE3 inhibition was completely ineffective in any experimental system tested, PDE4 inhibition markedly affected B lymphocyte proliferation (see Figure 4). It should be mentioned that similar results were obtained in experiments in which the influence of cyclic AMP elevating drugs on anti-CD40 Ab/IL-4-induced release of IL-6 from human B cells was investigated. PDE3 inhibition was ineffective whereas PDE4 inhibition resulted in an increase of IL-6 release, although the effect was less pronounced compared to the stimulation of proliferation. Again, db-cyclic AMP mimicked the effect of PDE4 inhibitors up to 100 μM, but neither PGE_2 nor forskolin significantly affected the release of IL-6 (data not shown).

In addition to the enhancing effect of PDE4 inhibitors on B cell growth, proliferating B cells displayed significantly lower PDE4 activity compared to resting cells (see Figure 6), most probably due to downregulation of PDE4 gene expression. This observation supports the concept that an increased cyclic AMP level due to diminished PDE4 activity promotes B lymphocyte proliferation under our experimental conditions. Two lines of evidence support the hypothesis of cyclic AMP as a critical regulator of B cell growth: first, the increased proliferative response induced by rolipram is paralleled by a rise in cyclic AMP and, secondly, PKA inhibitors not only abolished the PDE4 inhibitor effects as expected, but also strongly inhibited LPS/IL-4-induced DNA synthesis of control cells (see Figure 5). This suggests that a cyclic AMP signal is necessary to allow the initiation of DNA synthesis in human B lymphocytes.

Intriguingly, the different PKA inhibitors tested behaved oppositely on control cells and on cells coincubated with rolipram. Whereas KT 5720 more potently affected the proliferation in the presence of rolipram, 8-Br-Rp-cyclic AMPS more potently abrogated DNA synthesis under control conditions (see Figure 5). These effects imply a different mechanistical mode of PKA inhibition by these two agents.

PDE4 inhibitors are known to suppress a wide variety of inflammatory cell functions (reviewed in Dent & Giembycz, 1996) among which the antiproliferative effects on T lymphocytes have been extensively studied. In this respect, it was interesting to find an enhancement of polyclonal B cell proliferation in the presence of PDE4 inhibitors under our experimental conditions, emphasizing basic differences in the PDE profile and in PDE function between B and T lymphocytes. In addition, it is important to point out that earlier studies provided conclusive evidence for growth inhibiting effects by cyclic AMP elevating drugs on B cells (reviewed in Sanders, 1995). This suggests that a certain response triggered via the cyclic AMP/PKA pathway in B lymphocytes may be manifoldly influenced. These cyclic AMP signal modulators may include (i) the manner of stimulation, i.e. the nature of the B cell stimulus, (ii) the signal intensity and the duration of the cyclic AMP increase, (iii) the time point of measurement of a certain response, and (iv) the actual activation/differentiation status of the B cells under investigation.

For instance, it has been shown that β-adrenoceptor-mediated elevation of cyclic AMP inhibits the proliferation induced by LPS or anti-immunoglobulin antibodies. In contrast, activation of the same receptor enhances proliferation of B cells exposed to PMA/ionomycin (reviewed in

Sanders, 1995). Similar effects have been described for cholera toxin (CT), which leads to a cyclic AMP increase via activation of adenylyl cyclase. Whereas CT stimulated anti-IgM-activated tonsil B cell proliferation, CT selectively inhibited IL-2- but not IL-4-induced DNA synthesis (Garrone & Banchereau, 1993).

Our own data point to the importance of the cyclic AMP signal intensity in directing the fate of B cells. In accordance with experiments on human tonsillar B lymphocytes (Garrone & Bancherau, 1993), concentrations of db-cyclic AMP up to 100 μM increased polyclonally-induced DNA synthesis, whereas 1 mM of the same compound resulted in a strong inhibition of proliferation, presumably by the induction of cytotoxicity. The fact that mM concentrations of db-cyclic AMP have been shown to shorten the life span of human resting peripheral blood B cells *in vitro* by accelerating the onset of apoptosis (Lømo *et al*., 1995), reveals a mechanistic explanation for the latter observation and supports the concept of a bimodal cyclic AMP action on the control of B cell growth.

Furthermore, the time point of measuring the proliferative response might be critical. Data illustrated in the paper by Garrone and Banchereau (1993) showed that the kinetics of IL-4-dependent proliferation were significantly modified by agents increasing cyclic AMP, since CT, PGE_2 and forskolin partially inhibited the early [^{3}H]-thymidine uptake induced by IL-4 within 48 h, but strongly stimulated it when monitored after 3 or more days of culture. Thus, our data obtained with the PDE4 inhibitors rolipram and piclamilast are in accordance with this finding, since we investigated the role of cyclic AMP in the regulation of B lymphocyte proliferation in a 5 day experiment using the same costimulus (IL-4).

Finally, the effect of cyclic AMP on the regulation of proliferation may depend on the activation or differentiation level of B cells. For instance, cyclic AMP elevating agents were shown to increase synergistically DNA synthesis of B cells exposed to low-mitogenic anti-IgM Ab preparations, but did not enhance proliferation of B cells that were costimulated or pre-activated with mitogenic concentrations of the superantigen *Staphylococcus aureus*, strain cowan 1 (Anastassiou *et al*., 1992).

Intriguingly, PDE4 inhibitors were far more efficient than cyclic AMP analogues in enhancing B cell growth, although no other mechanism apart from elevation of cyclic AMP through the inhibition of its breakdown is known for PDE4 inhibitors. A theoretical explanation would be specific subcellular compartmentalization of PDE4 and PDE4 inhibitors at sites where a local increase in cyclic AMP triggers a proliferation enhancing signal. Since the use of cyclic AMP analogues always results in a net effect integrated over the whole cell, the results might differ not only quantitatively, but also qualitatively from the ones obtained with PDE4 inhibitors.

Surprisingly, we failed to find any effects of PGE_2 or forskolin on LPS/IL-4-induced proliferation, although other investigators have shown modulatory effects of both agents in human B cell systems (Garrone & Banchereau, 1993) and B cells, at least in the murine system (Fedyk *et al*., 1996), functionally coexpress a variety of prostaglandin receptors including the Gs-coupled EP_2 and EP_4 type.

In summary, the present paper describes the detailed PDE activity and mRNA expression pattern of human peripheral blood B lymphocytes, which substantially differs from the PDE profile of T lymphocytes, and underlines the prominent role of the PDE4 family in leucocytes. The direct comparison of normal and atopic cells reveals no evidence for a PDE dysregulation in B lymphocytes due to the atopy status. Furthermore, our data indicate that cyclic AMP/PKA-dependent signalling pathways are involved in the proliferative response of human B lymphocytes following stimulation with LPS/IL-4.

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