Identification of cyclic AMP-phosphodiesterase variants from the PDE4D gene expressed in human peripheral mononuclear cells

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Abstract To determine whether the expression of different PDE4D variants is unique to the rat or conserved through evolution, we have characterized the different PDE4D mRNAs expressed in human peripheral blood mononuclear cells. RT-PCR was performed using primers based on rat sequences and mRNAs from mononuclear cells. The specifically amplified fragments had a size identical to that predicted for rat PDE4D1, PDE4D2 and PDE4D3. Sequencing confirmed that these fragments are derived from the human PDE4D gene. Their sequence was highly homologous to that reported for the rat variants. cDNAs corresponding to the entire ORF of human PDE4D2 and PDE4D3 were expressed in mammalian cells, causing a large increase in PDE activity. Western blot analysis of human peripheral blood mononuclear cell extracts demonstrated the presence of proteins corresponding to the recombinant PDE4D1 and PDE4D2. The pattern of splicing and different promoter usage of the PDE4D gene is therefore conserved during evolution, which indicates an important physiological role.

Key words: Cyclic AMP; cAMP-phosphodiesterase; PDE4D gene; Human lymphocyte

1. Introduction

The intracellular levels of cAMP play a key role in the function of inflammatory and immune cells, since an increase in this second messenger suppresses a broad array of responses in these cells [1–3]. Activation of adenylyl cyclase [4–7] or inhibition of phosphodiesterases [8], the enzymes that hydrolyse cAMP, suppresses the proliferation of T lymphocytes. This cAMP-dependent inhibition is mediated by the type I PKA [9]. Immunocytochemistry and immunoprecipitation studies on T lymphocytes have shown that cAMP induces the translocation of type I PKA and promotes its interaction with the antigen specific T cell receptor-CD3 complex [10]. It has been hypothesized that a type I PKA-mediated phosphorylation may induce uncoupling of TCR-CD3 complex from the intracellular transduction pathways [10]. Additional early steps in T lymphocyte activation, such as stimulation of phospholipase C and tyrosine phosphorylation of the pp100 phosphoprotein, are inhibited by an increase in intracellular cAMP [11]. Finally, a role of cAMP in the inhibition of c-Jun N-terminal kinase has been recently demonstrated, indicating an additional crucial step in the pathway that causes inhibition of T lymphocyte proliferation [12].

Cyclic nucleotide phosphodiesterases [13,14], including the enzymes of the PDE4 family (cAMP-phosphodiesterases), control cAMP-levels in lymphocytes and monocytes, and therefore modulate cellular responses to different stimuli [15]. The identification of the PDE4 expressed in immune cells is of major interest from a therapeutical point of view, as well as for understanding immunological disorders [15].

Four PDE4 genes (PDE4A, PDE4B, PDE4C, PDE4D) are expressed in rat [16–19], mouse [20], and human [21–25], and their characterization has shown a close relationship between genes from two different species [14]. Furthermore, for three out of four rat and human genes, multiple mRNA variants and corresponding protein products have been detected [13,14]. The pattern of expression of multiple variants from the rat PDE4D gene [26,27] has shown that the heterogeneity of the different mRNAs lies in the 5′ region. The corresponding protein variants contain a common region encoded by exons 2 to 11 [26], and divergent amino-terminal regions derived from the assembly of different upstream exons [27]. The rat PDE4D1 and PDE4D2 mRNAs are generated by start sites controlled by an intronic promoter which is active in Sertoli and FRTL-5 cells [28]. These two variants differ in the alternate splicing of a short intron, producing a frameshift that affects the first in frame AUG codon [26]. Another variant, rat PDE4D3, detected in the brain and FRTL-5 cells, is derived from the activity of a different promoter and the transcription of upstream exons [27]. The human counterpart of this latter form has been described by Bolger et al. [22]. A fourth variant (PDE4D4) was also detected in human [22] and rat (Vicini, E. and Conti, M., unpublished results), suggesting the presence of a third, distinct promoter. Hormones control the activation and expression of the PDE4D variants through an increase in intracellular cAMP levels. PDE4D3 is activated by a PKA-mediated phosphorylation in FRTL-5 rat thyroid cells [27,29,30] and in U937 human monocytic cells [31]. Chronic hormonal stimulation induces the synthesis of rat PDE4D1 and PDE4D2 in FRTL5 cells [27,29] and Sertoli cells [32]. The expression of these two variants is important for the long term desensitization of these cells to hormones [30].

This study was undertaken to determine whether the multiplicity of variants arising from the PDE4D gene is conserved from rodents to humans during evolution and whether the PDE4D gene could take part in the regulation of cAMP levels in human peripheral blood mononuclear cells. For this purpose, we have characterized different PDE4D mRNAs and the corresponding proteins expressed in these cells by RT-PCR and Western blot analysis.

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Abbreviations: PDE, cyclic nucleotide phosphodiesterase; PKA, cAMP-dependent protein kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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2. Materials and methods

2.1. Cell isolation and rna preparation

Human mononuclear cells were prepared from the peripheral blood of healthy volunteers according to Boyum et al. [33], using Histopaque-1077 as the separating medium (Sigma). Poly(A)$^+$ RNA was purified by chromatography on oligo(dT)-cellulose using Pharmacia Quick Prep Micro mRNA purification columns. Total RNA was extracted using RNAzol (Tel-Test Inc.) according to the manufacturer's recommendations.

2.2. Polymerase chain reaction amplifications and Southern blot analysis

First-strand cDNA was generated using Pharmacia cDNA synthesis kit, according to the supplied protocol. 0.2 μg of poly(A)$^+$ RNA or 0.8 μg of total RNA were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using random hexadeoxynucleotides as primers. The complete first-strand cDNA reaction product was directly amplified by PCR. The primers used were oligonucleotide A, 5'-AATATGAAGGAGCAGCCC-3', corresponding to the 5' end sequence of rat PDE4D1 and rat PDE4D2; oligonucleotide B, 5'-GTACGGCAGGATGGCCCC-3', corresponding to a sequence in intron A of rat PDE4D1 [26]; oligonucleotide C, 5'-CCTCTTGAACTTGTTGGAGG-3', corresponding to the antisense sequence of rat PDE4D at the boundary between exons 2 and 3 [26]; oligonucleotide D, 5'-TGCCCACGATAGCTGCTCAAACAAG-3', corresponding to the 5' end sequence of rat PDE4D3; and oligonucleotide COM2, 5'-TCCAGACACCAGTCCAGCTCCTCCA-3', corresponding to the antisense sequence of rat PDE4D in a common region of the 3 variants. GenBank accession numbers for rat PDE4D1, PDE4D2, PDE4D3 sequences are: M25349, U09456, U09457. PCRs were performed in a volume of 50 μl, containing 50 mM KCl, 25 mM Tris-HCl pH 8.3, 2.4 mM $MgCl_2$, 0.01% gelatin, 10 pmol of each primer, 0.18 mM of each dNTP, and 2.5 units of Taq polymerase from Gibco BRL. The reactions were performed for 30 thermal cycles consisting of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C. An aliquot of PCR product was analyzed on 1.4% agarose gel in 90 mM Tris borate, 2.5 mM EDTA and stained with ethidium bromide. DNA bands were transferred onto a Nylon membrane (ICN), and the membrane was hybridized by using the oligonucleotide COM1 (5'-TGGCCAGTTTCTGGTAGGCCTCCTC-3') labelled with [γ-^{32}P]ATP (DuPont NEN) and T4 polynucleotide kinase (Promega), as previously described [34].

2.3. Subcloning and sequencing

The two DNA bands amplified with the oligonucleotides A and C (corresponding to the 5' ends of PDE4D1 and PDE4D2), and the band amplified with the oligonucleotides D and COM2 corresponding to the 5' end of PDE4D3, were purified from agarose gel by using Sephaglas BandPrep kit (Pharmacia), and subcloned into pUC18 vector using SureClone ligation kit (Pharmacia). The three DNA fragments were then sequenced using the TAQuence Cycle Sequencing kit (United States Biochemical).

2.4. PDE4D cDNA cloning

A cDNA containing the entire predicted ORF of human PDE4D2 was obtained by a single RT-PCR amplification using oligonucleotide A and oligonucleotide E, 5'-GCACTGTTACGTGTCAGGAGAACGATC-3', corresponding to the 3'-end antisense sequence of human PDE4D3 ([22], GenBank accession number: L20970). Amplification conditions were the same as above. The ~1.7 kb fragment obtained was inserted in the pUC18 vector at the *Sma*I site, and was then released from the vector using *Eco*RI and *Xba*I. The PDE4D2 cDNA was then inserted in the pCMV5 expression vector at the *Eco*RI and *Xba*I sites of the polylinker. A cDNA encoding the entire human PDE4D3 ORF was constructed by ligating a fragment corresponding to the specific PDE4D3 5' end and a fragment corresponding to the PDE4D common region, taking advantage of a unique *Stu*I site adjacent to the splicing boundary common to the three PDE4D variants. For this purpose, the PCR-amplified PDE4D3 5' end inserted in pUC18 vector was released using *Eco*RI and *Stu*I. A frag-

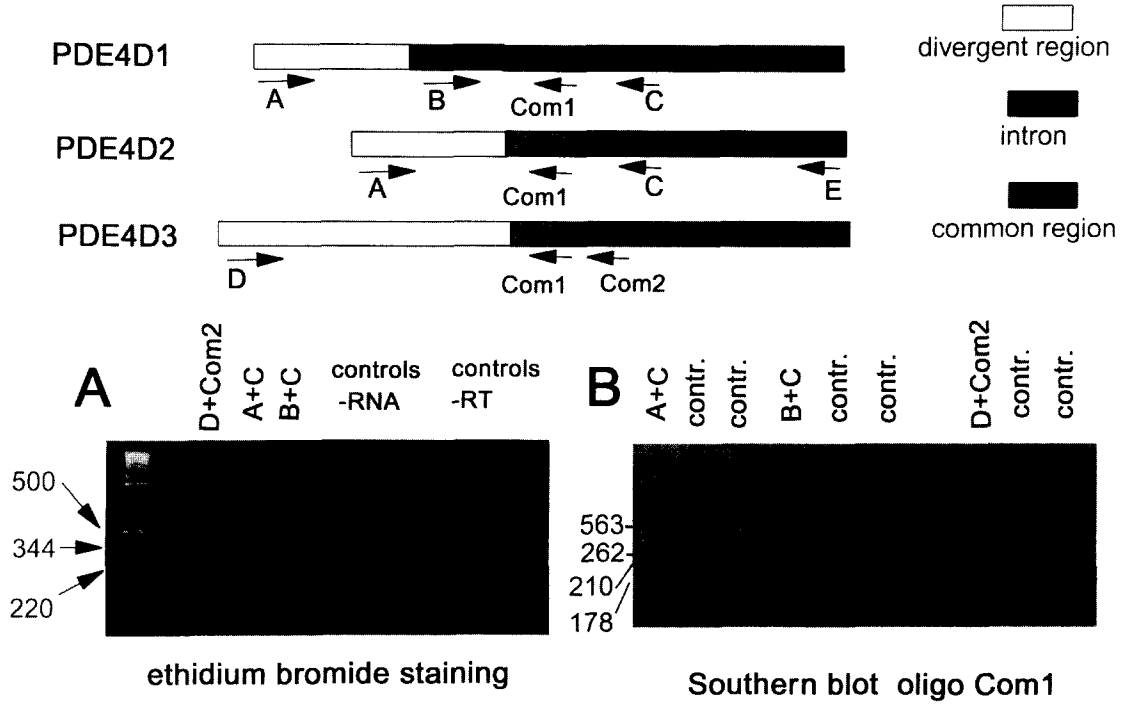

Fig. 1. RT-PCR amplification of the 5' ends of the different human PDE4D variant cDNAs. The primer pair A+C is specific to the PDE4D1 and PDE4D2 cDNAs. The primer pair B+C is specific to PDE4D1 cDNA. The primer pair D+COM2 is specific to PDE4D3 cDNA. The control amplifications were performed in the absence of mRNA (−RNA), or in the absence of reverse transcriptase in the cDNA synthesis step (−RT). The amplification products were electrophoresed on agarose gel, and stained with ethidium bromide. After transfer to membrane, the specificity of amplification was assessed by hybridization with the radiolabeled oligonucleotide COM1. Ethidium bromide staining revealed three amplified bands with primers D+COM2. Only the band of the expected size (563 bp) hybridized with COM1. With the other primer pairs, the size of the observed bands matched the ones expected on the basis of rat sequences (A+C: 175 and 261 bp; B+C: 208 bp).

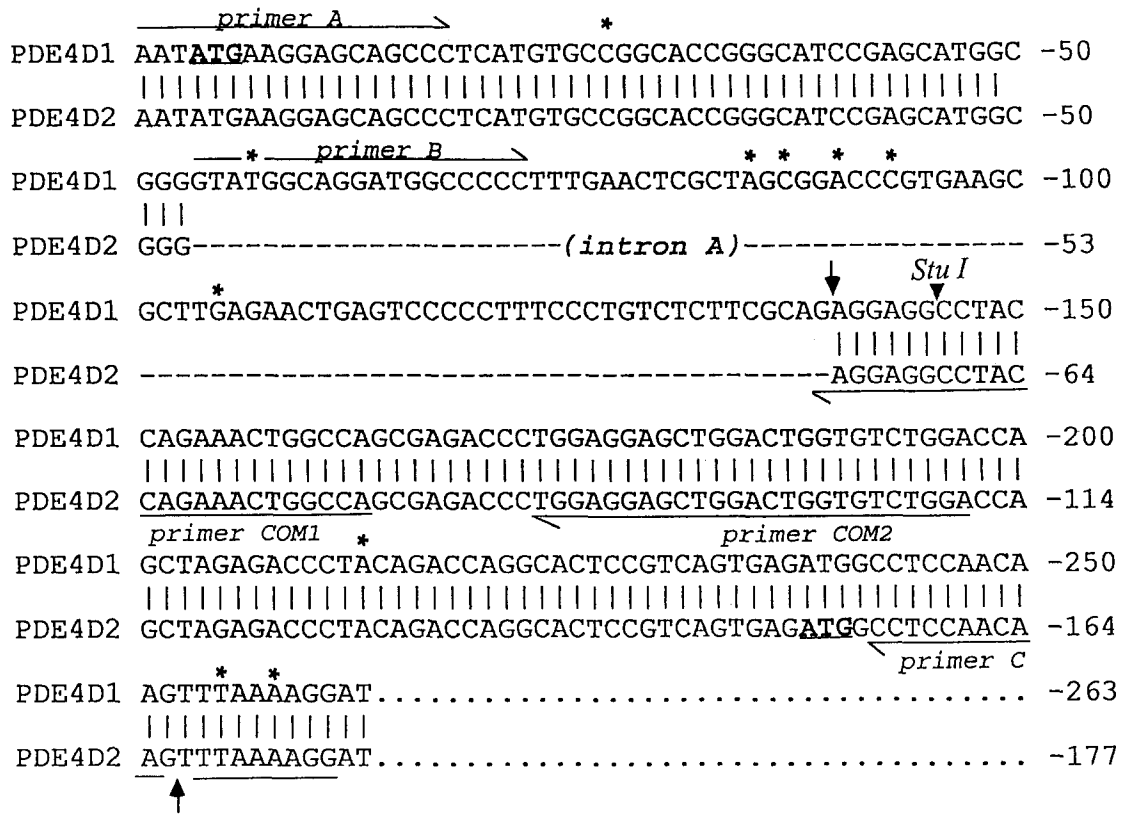

Fig. 2. Nucleotide sequence of the 5′ ends of human PDE4D1 and human PDE4D2 cDNAs amplified by RT-PCR from human blood mononuclear cell mRNA. The entire sequences of the fragments amplified with the primer pair A+C are shown. The position of intron A [26] is indicated by a dashed line in the PDE4D2 sequence. Asterisks indicate a nucleotide different from rat sequence. The putative initiation ATG codons for PDE4D1 and PDE4D2 are indicated by bold characters. The point of divergence between PDE4D1, PDE4D2 and PDE4D3 mRNAs (5′ boundary of exon 2 [26]) is indicated by a downward arrow. The putative boundary between exons 2 and 3 [26] is indicated by an upward arrow. The position of a *Stu*I site common to all three PDE4D variants and used for construction of PDE4D3 cDNA is shown.

ment corresponding to the region common to the three PDE4D variants was obtained from the PDE4D2-pUC18 construct above-described using *Stu*I and *Xba*I. These two fragments were ligated together and inserted in the pCMV5 expression vector at *Eco*RI and *Xba*I sites. The complete sequences of the cDNAs were determined by the dideoxy chain termination method of Sanger, or by automatic sequencer (Beckman Center, Stanford University). The sequences of human PDE4D1, PDE4D2, PDE4D3 have been deposited in GenBank under the following accession numbers: U50157, U50158, U50159.

2.5. Transfection of 293 cells

Human kidney 293 cells were transfected using the calcium phosphate method, with 10–20 μg of pCMV5 human PDE4D2 or pCMV5 human PDE4D3 plasmid per 10 cm dish, as previously described [34].

2.6. Western blot analysis

The soluble extracts from transfected cells were immunoprecipitated by an anti-cAMP-PDE antiserum (K116) complexed to Pansorbin, as previously detailed [29]. K116 antiserum was raised against a peptide P2224 located at amino acid positions 105–126 of PDE4D1 and recognizes the different PDE4A, PDE4B, PDE4D isoforms [32]. Immunoadsorbed proteins were solubilized in 1% SDS in phosphate-buffered saline, subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore) as described previously [29]. The cytosolic fraction of human blood mononuclear cells (approx. 5×10^8 cells) was fractionated on a Mono Q HPLC column (Pharmacia) by using a 0.2–0.4 M NaCl gradient in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 7 mM 2-mercaptoethanol, pH 7.5. The phosphodiesterase activity of the fractions was measured as reported in [35] with 1 μM cAMP as substrate. The active fractions which were inhibited by 10^{-5} M rolipram were precipitated by trichloroacetic acid. For this purpose, 15 μl of 1% deoxycholate and 100 μl of 72% TCA were added per ml of eluate. After 30 min at 4°C, the pellets were neutralized with minute volumes of 1 M Tris. Alternatively, cAMP-PDEs present in human blood mononuclear cell extracts were immunoprecipitated as above. In both cases, proteins were subjected to SDS-PAGE and transferred to Immobilon membranes. Western blot analyses were performed by using as primary antibody either the anti-PDE4 antiserum K116 or the anti-PDE4D monoclonal antibody M3S1 as previously described [29]. M3S1 was raised against a fusion protein obtained by linking the glutathione transferase to the carboxy terminal region of rat PDE4D and specifically recognizes the PDE4D isoforms, as detailed in [36]. The immunoreactive bands were detected by an enhanced chemiluminescence method, following the manufacturer's protocol (Amersham).

3. Results and discussion

Oligodeoxynucleotide primers designed according to the 5′ coding regions of the previously described rat PDE4D1, PDE4D2, PDE4D3 cDNAs were used to amplify mRNA from blood mononuclear cells of human donors. PCR amplification yielded fragments of the appropriate size, suggesting the expression of the human counterparts of the three rat PDE4D mRNAs in these cells (Fig. 1A). The primer pair A+C yielded the amplification of two DNA fragments, which fit the model proposed for the rat PDE4D1 and PDE4D2 mRNA variants [26,27], the larger fragment corresponding to the rat variant PDE4D1 (with intron A maintained) and the smaller fragment corresponding to the rat variant PDE4D2 (with intron A spliced out). The presence of a se-

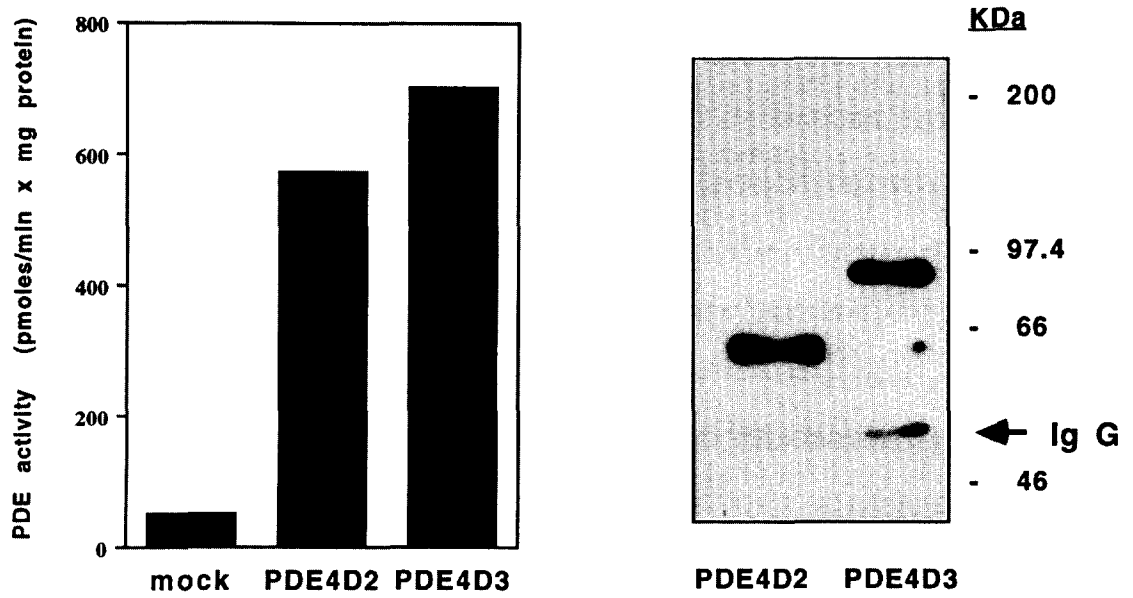

Fig. 3. Expression of human PDE4D2 and human PDE4D3 in transfected 293 cells. Full length PDE4D2 and PDE4D3 cDNAs were subcloned in the eukaryotic expression vector pCMV5. 10–20 μg of pCMV5-PDE4D2 or pCMV5-PDE4D3 constructs were transfected into 293 cells. (A) PDE activity of the control and PDE-transfected cell cytosolic extracts. (B) Western blot analysis of the cytosolic extract from PDE-transfected cells: cyclic AMP-PDEs in the cell extracts were immunoprecipitated as described in section 2, and subjected to Western blot analysis by using the PDE4D-specific antibody M3S1.

quence corresponding to intron A in a human mRNA was confirmed by using the primers B+C. The sequence of oligonucleotide B is included in intron A, and the only amplified fragment corresponds, in this case, to the 5′ end of the human counterpart of rat PDE4D1 mRNA (Fig. 1A). The major band amplified with the primers D+COM2 corresponds to the amplification of the human PDE4D3 mRNA 5′ end. The specificity of the amplifications was assessed by hybridization of the DNA fragments to an internal oligonucleotide (Fig. 1B). The amplification of contaminating genomic DNA could be excluded by omission of reverse transcriptase and, in some cases, by using oligonucleotide C, which overlaps the boundary between exons 2 and 3. This primer can only anneal to the sequence corresponding to a mature mRNA with the intron between exons 2 and 3 spliced out [26].

The two DNA fragments amplified by primers A+C were subcloned and sequenced. The fragments showed high homology with the corresponding 5′ end sequences of rat PDE4D1 and rat PDE4D2 (Fig. 2). However, several base differences between human and rat sequences were observed, ruling out the possibility that an artifactual amplification of contaminants had occurred. The differences were mainly clustered in the intron A sequence. The sequence of the fragment amplified with the primers D+COM2 was identical to the human PDE4D3 sequence previously reported by others [22].

To obtain full length PDE4D2 cDNA, human blood mononuclear cell mRNA was amplified using the primers A+E as described in section 2. The ~1.7 kb fragment obtained was subcloned in the pCMV5 expression vector and sequenced. The amplified sequence included the entire ORF of human PDE4D2. A cDNA containing the entire ORF of human PDE4D3 was constructed by ligating an *Eco*RI-*Stu*I fragment, corresponding to the PDE4D3 specific 5′ end sequence, to a *Stu*I-*Xba*I fragment corresponding to the sequence common to all PDE4D variants. This cDNA was subcloned into the *Eco*RI and *Xba*I sites of pCMV5 vector polylinker and the complete sequence of the construct was determined. To verify whether the ORF present in these two cDNAs encoded active proteins, the corresponding expression vectors were used to transfect human kidney 293 cells. Both human PDE4D2 and PDE4D3 cDNAs induced the expression of high levels of PDE activity in transfected cells (Fig. 3). Western blot analysis, with a monoclonal anti-PDE4D antibody (M3S1), indicated the appearance of a 67 kDa protein in cells transfected with PDE4D2 cDNA, and of a 93 kDa protein in cells transfected with PDE4D3 cDNA (Fig. 3). Therefore, recombinant human PDE4D2 and PDE4D3 proteins have molecular weights identical to those previously determined for the corresponding rat forms [27].

The cAMP phosphodiesterases expressed in human mononuclear cells, prepared from the blood of different healthy donors, were analyzed by Western blot using a polyclonal anti-PDE4 antibody (K116) and the monoclonal anti-PDE4D antibody M3S1 (Fig. 4). Although some differences from sample to sample were noticed, Western blot analysis of seven independent samples with antibody M3S1 indicated a predominant polypeptide of 72 kDa, corresponding to the molecular weight of PDE4D1, and a less abundant polypeptide of 67 kDa, corresponding to the molecular weight of PDE4D2. Some unidentified polypeptides with a size smaller than PDE4D2 were also recognized. Among these bands, only the 72 kDa band, corresponding to PDE4D1, was recognized by the anti-PDE4 K116. It is possible that expression of PDE4D2 is too low in these cells to be detected by this latter antibody. The anti-PDE4 K116 also recognized a band of 120 kDa, which is not detected by M3S1, indicating that it is not a PDE4D isoform. The molecular weight of this PDE is similar to that predicted for PDE4A5 [22], and a band of similar size was recently detected in human U937 cells by using an anti-PDE4A antibody [37].

These data demonstrate that short PDE4D variants are expressed in human blood mononuclear cells. In the rat, expres-

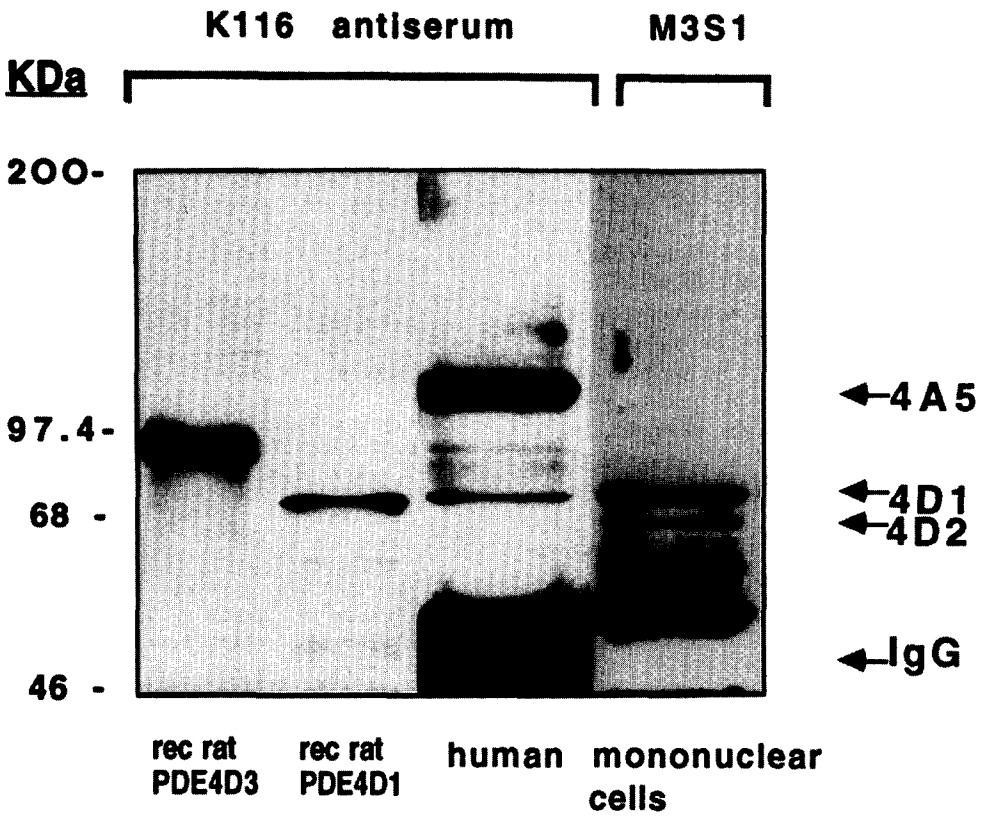

Fig. 4. Western blot analysis of cytosolic extract from human blood mononuclear cells. Cyclic AMP-PDEs in the cell extract were either immunoprecipitated or partially purified by HPLC as described in section 2, and analyzed by Western blotting using either the anti-PDE4 non-selective antiserum K116, or the PDE4D-specific monoclonal antibody M3S1. This figure is representative of 7 independent experiments performed with different donors. Recombinant rat PDE4D1 and PDE4D3 proteins were loaded in parallel for comparison.
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sion of these variants is under the control of an internal promoter regulated by cAMP [27,28]. Activation of this promoter and expression of the PDE4D1 and PDE4D2 variants are involved in the long-term adaptation and desensitization of target cells to hormones. Our findings suggest that a similar feedback loop could play a role in the control of intracellular cAMP levels in human blood mononuclear cells. This hypothesis is in agreement with a recent report describing the induction of proteins very similar to PDE4D1 and PDE4D2 by cAMP in a human promonocytic cell line [38]. Furthermore, it has been shown that, in the rat, the cAMP regulation of the activity of the long PDE4D3 variant occurs at the post-translational level, via a cAMP-dependent phosphorylation [27,29,30]. Studies with human U937 cells point to the absence of up-regulation of this variant by cAMP at the transcriptional level [37]. Moreover, in the same cell type, PDE4D3 undergoes a short-term regulation by PKA-phosphorylation [31], which strongly suggests that its regulation is similar in human and rat. It is worth remarking that the pattern of splicing at the boundary of exon 2 has been conserved from rodents to humans, suggesting a physiological relevance for the differential regulation of protein variants arising from the PDE4D gene [27].

The newly identified human PDE4D1 and PDE4D2 lack the so-called UCR I (upstream conserved region I), a region of high homology with the drosophila dunce PDE sequence, which is present at the N-terminus in human and rat PDE4D3 and other PDE4 variants [22,27,14]. Its strong conservation through evolution suggests an important function for this sequence [22]. However, the existence of PDEs lacking this region, in both rat and human, indicates that it is not essential for catalysis. The UCR I region may instead play a role in the regulation of enzyme activity. Indeed, consensus sites for PKA-mediated phosphorylation in rat PDE4D3 reside within this region [39].

Our data indicate that, although the PDE4D3 mRNA is expressed in human blood mononuclear cells, the corresponding protein could not be detected with two different anti-PDE4 specific antibodies. This can be explained by a low translation rate of the PDE4D3 message, or by an expression restricted to a minor cell type, or alternatively by instability of the protein. The other PDE4D isoforms, and mainly PDE4D1, were present in amounts sufficient to be detected. A major isoform expressed in these cells can probably be identified as the product of a different gene, PDE4A5.

Since blood mononuclear cells are mostly constituted of lymphocytes (85–90%), the pattern of PDE4 determined in the present study can be considered as representative of normal human lymphocytes. In contrast to our results, a previous study has shown that in a human leukemic lymphocyte line expression of PDE4D3 mRNA could only be detected after dibutyryl cAMP treatment of the cells [40]. The presence of variants PDE4D1 and PDE4D2 was not addressed. However, it must be emphasized that leukemic transformation is known to be accompanied by an abnormal PDE expression [8]. Therefore, the PDE pattern of transformed lymphocytes

may not be representative of the actual repertoire of normal cells.

In addition to lymphocytes, human blood mononuclear cells include a significant percentage of monocytes, that may participate in the PDE pattern we have determined. Previous studies on promonocytic cell lines pointed to the expression of PDE4D3 in unstimulated U937 cells [31,37,40], and to its absence in Mono Mac 6 cells [38]. The expression of other isoforms, namely PDE4D1, PDE4B2, and PDE4A5, was induced by cAMP-elevating agents in these cell lines [37,38,40]. Thus, there remains considerable uncertainty concerning the forms expressed in unstimulated normal human monocytes, and it is difficult to completely rule out the participation of this cell type to the isoenzyme expression pattern we have determined from blood mononuclear cells. However, important differences between lymphocyte and monocyte PDE4 profiles are suggested, mainly concerning the isoforms encoded by genes PDE4B and PDE4D. Confirmation of this difference could open new avenues for selective pharmacological actions on a given cell type, by using isoenzyme-specific inhibitors.

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