Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene

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We have isolated and characterized complete cDNAs for two isoforms (HSPDE4D4 and HSPDE4A5) encoded by the human *PDE4D* gene, one of four genes that encode cAMP-specific rolipram-inhibited 3',5'-cyclic nucleotide phosphodiesterases (type IV PDEs; PDE4 family). The HSPDE4D4 and HSPDE4D5 cDNAs encode proteins of 810 and 746 amino acids respectively. A comparison of the nucleotide sequences of these two cDNAs with those encoding the three other human PDE4D proteins (HSPDE4D1, HSPDE4D2 and HSPDE4D3) demonstrates that each corresponding mRNA transcript has a unique region of sequence at or near its 5'-end, consistent with alternative mRNA splicing. Transient expression of the five cDNAs in monkey COS-7 cells produced proteins of apparent molecular mass under denaturing conditions of 68, 68, 95, 119 and 105 kDa for isoforms HSPDE4D1–5 respectively. Immunoblotting of human cell lines

and rat brain demonstrated the presence of species that co-migrated with the proteins produced in COS-7 cells. COS-cell-expressed and native HSPDE4D1 and HSPDE4D2 were found to exist only in the cytosol, whereas HSPDE4D3, HSPDE4D4 and HSPDE4D5 were found in both cytosolic and particulate fractions. The IC_{50} values for the selective PDE4 inhibitor rolipram for the cytosolic forms of the five enzymes were similar (0.05–0.14 μ M), whereas they were 2–7-fold higher for the particulate forms of HSPDE4D3 and HSPDE4D5 (0.32 and 0.59 μ M respectively), than for the corresponding cytosolic forms. Our data indicate that the N-terminal regions of the HSPDE4D3, HSPDE4D4 and HSPDE4D5 proteins, which are derived from alternatively spliced regions of their mRNAs, are important in determining their subcellular localization, activity and differential sensitivity to inhibitors.

INTRODUCTION

The cAMP-specific phosphodiesterases (PDEs) are a distinct family of 3',5'-cyclic nucleotide PDEs, characterized by a high specificity and affinity for cAMP and by their ability to be inhibited by a specific class of compounds, including the antidepressant drug rolipram [1–3]. They are the mammalian homologues of the *dunce* gene of *Drosophila melanogaster*, implicated in learning and memory [4–6]. The cAMP-specific PDE proteins are encoded by four genes in mammals (*PDE4A*, *PDE4B*, *PDE4C* and *PDE4D*). At least three of these genes encode multiple PDE isoforms, encoded by different alternatively spliced mRNA transcripts (reviewed in [1]). The physiological implications of this diversity of cAMP-specific PDE isoforms is not known, but many of the isoforms differ in their biochemical properties, phosphorylation, intracellular targeting, protein–protein interactions and patterns of expression in tissues [1–3]. In addition, the amino acid sequences of many of the different cAMP-specific PDE isoforms are strongly conserved among different mammalian species, suggesting that their physiological functions may also be similar.

We and others have previously isolated cDNA clones derived from different mRNA transcripts of the human and rat *PDE4D* genes, which have a structure consistent with alternative mRNA splicing. In rats, three different transcripts from this gene have been reported [7–11]: PDE4D1 (formerly known as ratPDE3.1), PDE4D2 (formerly known as ratPDE3.2) and PDE4D3 (formerly

known as ratPDE3.3). We have reported previously [12] the cloning of two human *PDE4D* mRNA transcripts: PDE4D3 and PDE4D4. The cDNAs representing the human PDE4D1 and PDE4D2 transcripts have also been reported [13]. A comparison of the sequences of the rat and human PDE4D1, PDE4D2 and PDE4D3 proteins reveals them to be highly conserved: each of the human proteins is nearly identical in length with its rat counterpart and has greater than 90% amino acid identity. The significance of this diversity of PDEs is not known, but previously published data have suggested that each of the various isoforms has a distinct function. For example, the rat PDE4D1, PDE4D2 and PDE4D3 transcripts have different patterns of distribution in tissues [11], and differ in their ability to be induced by changes in cAMP levels [7,14,15]. The rat PDE4D1, PDE4D2 and PDE4D3 proteins differ in their enzymological properties and in their regulation by phosphorylation [9,15].

In order to characterize more fully the diversity of proteins encoded by the *PDE4D* gene, we have isolated cDNAs representing additional mRNA transcripts from the human gene and shown that they encode major PDE4D species present natively in cells and tissues. The structure of these cDNAs demonstrate that transcripts from the *PDE4D* gene undergo a complex pattern of alternative splicing, which resembles that of transcripts from the *D. melanogaster dunce* gene. Our data demonstrate that the *PDE4D* gene has a more complex structure than that reported previously [13,16]. We have also studied the biochemical properties of the various PDE4D isoforms, and for this purpose

Abbreviations used: ORF, open reading frame; PDE, cyclic nucleotide phosphodiesterase; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; RT-PCR, reverse transcriptase PCR; UCR, upstream conserved region; GST, glutathione S-transferase.

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utilized a single system, expression in mammalian COS-7 cells, in which the properties of the five individual isoforms could be compared. We demonstrate that the proteins encoded by the various transcripts differ in their ability to hydrolyse cAMP, to be inhibited by rolipram and in their subcellular localization.

EXPERIMENTAL

Materials

A HeLa cell line (human cervical carcinoma; subclone D98/AH-2) cDNA library, cloned into the *EcoRI*–*XhoI* sites of the Lambda uni-ZAP vector [17], was obtained from Stratagene. A Manca human B-cell lymphoma line [18] cDNA library, cloned into the *EcoRI* site of Lambda gt10 [19], was from Dr. P. Snyder, (ICOS Corporation, Bothwell, WA, U.S.A.). All other reagents were as described previously [21].

Isolation and analysis of cDNA clones

Procedures were as described previously [19], unless otherwise specified. The HeLa cDNA library was screened with a hybridization probe corresponding to amino acids 134–181 of the pPDE43 cDNA (human PDE4D3 isoform; Figures 1 and 2), which was labelled by random-priming. Hybridization was carried out at 65 °C in 6 × SSC/0.5% SDS, and the final wash at 60 °C in 0.3 × SSC/0.1% SDS. The Manca cDNA library was screened with a ³²P end-labelled oligonucleotide (5'-CAGAAT-AGCAGAGTTGTCTGGTAACCGGCCCTTGACTGTT-3'), and subsequent characterization of the pDun411 clone (see below) was performed as described for the isolation of PDE1 cDNAs [20]. cDNA clones were sequenced on both strands by dye-cycle sequencing using an ABI prism sequencer (Perkin-Elmer). Amino acid sequences were aligned by using the Genalign program (Intelligenetics [22]).

RNase-protection analysis

Total RNA was isolated from various human cell lines by the guanidine thiocyanate/CsCl method [23]. All the cell lines used in this study were obtained from the American Type Culture Collection and are described in their catalogue and on their World Wide Web site. A single-stranded antisense probe was generated from nucleotides 1–320 of pPDE79 cloned into

pGEM3zf (Promega). RNase-protection assays were performed as described [12], with 10 µg of RNA per reaction. RNase digestions were carried out for 45 min at 30 °C with 2 units/reaction RNase ONE (Promega), using the conditions recommended by the manufacturer. Gels (6% acrylamide, 7 M urea, 1 × 0.089 M Tris/borate/0.002 M EDTA, pH 8.0) were autoradiographed at –70 °C for 2 days with intensifying screens.

Generation and expression of mammalian cell expression vectors

Constructs were prepared containing the full open reading frame (ORF) of pPDE39, pPDE43, pPDE79, pDun411 or pPDE82 cloned into the *NotI* site of pcDNA3 (Invitrogen). In these constructs, the insert is expressed under the control of the cytomegalovirus intermediate early gene promoter. These constructs were prepared by the addition of *NotI* sites to the cDNAs by the use of PCR, as described previously [12].

Cell lines and culture conditions

All cell lines used in this study were obtained from the American Type Culture Collection. The lines were grown in Dulbecco's modified Eagle's medium, supplemented with fetal calf serum and antibiotics. Transfection of monkey COS-7 cells (hereafter called COS cells) was performed as described previously [24].

Generation and characterization of monoclonal antibodies

A glutathione S-transferase (GST) fusion protein containing the C-terminal 65 amino acids of the PDE4D3 protein was generated using the vector pGEX2T (Promega). This sequence corresponds to a portion of the C-terminal region common to all of the known human PDE4D proteins (see Figure 2). Mice were immunized, hybridomas were generated and the PDE4D-selective hybridoma 61D10E was cloned using standard methods [25]. The 61D10E monoclonal antibody used for these studies was purified from ascites fluid with Protein A–Sepharose 4 Fast Flow (Pharmacia).

Generation and characterization of polyclonal antibodies

A GST fusion protein containing the C-terminal 40 amino acids of PDE4D2 was generated using the vector pGEX-3X-1 (Promega) and called GST-CT(1–40)4D. This sequence corresponds to a portion of the C-terminal region common to all of the known human PDE4D proteins (see Figure 2). This fusion protein was used to raise and purify polyclonal antibodies as described before by us [26] for PDE4A.

In immunocompetition studies (carried out as described previously for PDE4A antibodies [26]) recognition of PDE4D forms by the polyclonal antibody was blocked by treatment with either the immunogen GST-CT(1–40)4D or by GST-CT(1–66)4D but not by GST itself. For the monoclonal antibody, recognition of PDE4D forms was blocked by GST-CT(1–66)4D (see the Results section) but not by treatment with either GST or GST-CT(1–40)4D (results not shown). This signifies that the epitope in PDE4D recognized by the monoclonal antibody 61D10E was within a region 40–66 residues from the C-terminus of PDE4D3.

Generation of an epitope-tagged PDE4D3 construct

Two 5'-phosphorylated complementary oligonucleotides coding for the 11-amino acid VSV-epitope [27] followed by a translation stop signal were constructed to generate overhanging *AgeI* and *SalI* restriction sites after hybridization. This DNA fragment was subcloned in-frame into an *AgeI*–*SalI*-digested pCI-plasmid into

Figure 1 Structure of mRNA transcripts from the human *PDE4D* gene

The numbers indicate transcripts represented by the following cDNAs: 1, pDun411 (PDE4D1); 2, pPDE82 (PDE4D2); 3, pPDE43 (PDE4D3); 4, pPDE39 (PDE4D4); 5, pPDE79 (PDE4D5). The heavy bar indicates sequences common to *D. melanogaster* and mammalian cAMP-specific PDEs, which contain the shared regions [upstream conserved region 1 (UCR1), UCR2 and the catalytic region (catalytic)], as indicated by the cross-hatched regions. Thinner lines indicate sequence regions unique to each cDNA. The lines merge where the sequences of the cDNAs join that of the shared sequence. The dashed line connecting transcripts 1 and 2 indicates the region of sequence in PDE4D1 that is spliced out in PDE4D2. Small dark boxes indicate initiation codons, and the asterisk indicates the common termination codon. The initiation codon of the pPDE82 cDNA is located within UCR2. The arrows labelled A and B indicate the two major points of alternative splicing of the transcripts.

Figure 2 Alignment of the amino acid sequences of the N-terminal regions of the proteins encoded by (1) pDun411 (PDE4D1), (2) pPDE82 (PDE4D2), (3) pPDE43 (PDE4D3), (4) pPDE39 (PDE4D4) and (5) pPDE79 (PDE4D5)

The location of the putative initiation methionine of the PDE4D2 protein is indicated by (2). Underlined regions indicate UCR1, UCR2 and the approximate extent of the catalytic region of the PDEs. Only the N-terminal region of the alignment is shown, as the remaining sequences of all the splice variants are identical with that of the corresponding regions of the protein encoded by pPDE43, as described previously [12].

which PDE4D3 had been subcloned. This yielded the expression plasmid pCI-HSPDE4D3-VSV. The VSV epitope-tagged PDE-4D3 was detected using a monoclonal antibody [27].

Reverse transcriptase (RT)-PCR analysis

This was performed on rat brain RNA using primers and methodology previously described in detail [28].

SDS/PAGE and immunoblotting

These were as described previously [24,29].

Preparation and fractionation of tissue homogenates

Homogenates from cultured cell lines or rat brain were prepared as described previously [24,30]. These homogenates were then fractionated as follows. For the low-speed pellet (P1), they were centrifuged at 1000 g_{av} for 10 min. The supernatant from this step was then centrifuged at 100000 g_{av} for 1 h to yield a high-

speed pellet (P2, particulate) fraction, and a supernatant (cytosolic) fraction.

Treatment of high-speed pellet fractions with salt or detergent

High-speed pellet preparations (0.2 mg) were treated with KHEM buffer (50 mM KCl, 50 mM Hepes/KOH, 10 mM EGTA, 1.92 mM $MgCl_2$, pH 7.2) containing the indicated NaCl or Triton X-100 concentrations respectively. They were then incubated on ice for 30 min, and then centrifuged at 100000 g_{av} for 1 h at 4 °C. The resulting pellet was resuspended in KHEM plus the appropriate NaCl or Triton X-100 concentration, and the pellet and supernatant were then analysed by immunoblotting.

PDE assays

PDE activity was assayed by a modification of the procedure of Thompson and Appleman [31] and Rutten et al. [32], as described previously [33]. All assays were conducted at 30 °C, and in all experiments a freshly prepared slurry of Dowex/water/ethanol (1:1:1, by vol.) was used. In all experiments, initial rates were taken from linear time courses of activity. K_m values were determined over a substrate range of 0.25–25 μM cAMP (seven different concentrations). Dose-dependent inhibition by rolipram was determined in the presence of 1 μM cAMP and over a range (eight to ten different values) of 10 nM to 100 μM rolipram. The IC_{50} was then determined from these values, using a least-squares fitting algorithm. Rolipram was dissolved in 100% DMSO as a 1 mM stock and diluted in 20 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$ to provide a range of concentrations in the assay. The residual levels of DMSO were shown not to affect PDE activity over the ranges used in this study. Protein concentrations were measured by the method of Bradford, using BSA as standard [34].

Relative V_{max} determinations

These were performed as described previously [21,24,35]. Briefly, increasing concentrations of high-speed pellet protein from COS cells, transfected with various PDE-expressing plasmids, were analysed by immunoblotting. Either an ^{35}S -labelled secondary (anti-mouse) antibody or an enhanced chemiluminescence procedure (Amersham) was used as a detection system. For ^{35}S -labelling, a phosphorimager was used for detection. For enhanced chemiluminescence, the blots were scanned. The appropriate signal intensity was plotted against mg of sample protein to yield a linear graph, and used to determine the relative amounts of PDE protein in each of the preparations. For the V_{max} determinations, amounts of membrane protein that would provide equal amounts of PDE protein were used in the PDE assays. To define K_m values, data from PDE assays were analysed by computer fitting to the hyperbolic form of the Michaelis–Menten equation using an iterative least-squares procedure (Ultrafit; with Marquardt algorithm, robust fit, experimental errors supplied; Biosoft). Relative V_{max} values could be calculated using the Michaelis equation and the experimentally derived K_m values, as described previously [35].

Statistical methods

All P values were determined by Student's t test.

RESULTS

Isolation of cDNA clones for additional human PDE4D mRNAs

We have reported previously the cloning of the human *PDE4D* cDNAs, pPDE43 and pPDE39, from a human fetal brain cDNA

Table 1 cDNA clones corresponding to transcripts from the human *PDE4D* locus and properties of their corresponding proteins

The clones are identified with their locus (isoform) names and GenBank accession numbers. Accession numbers with an asterisk (*) correspond to the clones of Nemoz et al. [13]. All other accession numbers are from Nemoz et al. [13], or the present study. The letters HS, for *homo sapiens*, are usually prefixed to the isoform name (e.g. HSPDE4D1), but are omitted for clarity in the Table and the text. Also shown are the predicted sizes of the five proteins and their apparent molecular masses under denaturing conditions. To determine the apparent molecular masses of the proteins, homogenates from COS cells expressing the five plasmids were subjected to SDS/PAGE and immunoblotted, as described in the legend to Figure 3 (top).

Plasmid name	Isoform name	Number of amino acids	Accession number	Predicted size (kDa)	Observed size (kDa)
pDun411	PDE4D1	586	U50157*, U79571	66.5	68 ± 1
pPDE82	PDE4D2	508	U50158*, AF012074	57.8	68 ± 1
pPDE43	PDE4D3	673	L20970, U50159*	76.5	95 ± 2
pPDE39	PDE4D4	810	L20969	91.1	119 ± 2
pPDE79	PDE4D5	746	AF012073	84.4	105 ± 2

library [12]. pPDE43 encodes the PDE4D3 protein, which is 673 amino acids in length (Table 1, Figures 1 and 2). As part of the present study, we resequenced pPDE39 and additional clones from the fetal brain library which completely overlapped its ORF. This demonstrated that the pPDE39 ORF extends considerably more 5' than we reported previously [12]. The revised pPDE39 ORF encodes a protein, called PDE4D4, of 810 amino acids, rather than the 699 reported previously. Of these 810 amino acids, 658 are identical with the corresponding region of PDE4D3, and 152, located at the N-terminus, are different (Figure 2).

To search for additional mRNA splice variants encoded by the human *PDE4D* gene, probes derived from various regions of *PDE4D* transcripts were used to screen cDNA libraries derived from two different human cell lines. The probes used in these screens (see the Experimental section) were designed to detect clones that corresponded to all the rat and human *PDE4D* mRNAs that had been isolated to date [7–9,11,12,36]. Multiple different cDNA clones, derived from two different mRNAs, were isolated from a HeLa cell line cDNA library. The first of these mRNAs, corresponding to the pPDE79 cDNA (Figure 1), contained one large ORF, which was confirmed over its entire length by at least one other overlapping cDNA clone. The pPDE79 mRNA encodes a protein of 746 amino acids, of which all but 88, located at the extreme N-terminus, are contained in PDE4D3 (Figure 2). The second of the HeLa-cell mRNAs, corresponding to the pPDE82 cDNA, contains an ORF of 508 amino acids, all of which are contained in PDE4D3 (Figure 2). Screening of a Manca cell line cDNA library yielded a third cDNA, pDun411. pDun411 contained one ORF of 586 amino acids, of which all but 45 are contained in PDE4D3 (Figure 2). The putative start codons of the pPDE39, pPDE43, pPDE79, pDun411 and pPDE82 ORFs are preceded by upstream termination codons in all three frames, although they only weakly satisfy the criteria of Kozak [37].

Some of the cDNAs that we have isolated have been reported, at least in part, by others (Table 1). For example, the deduced amino acid sequences of the proteins encoded by pDun411 and pPDE82 are identical with those of the human PDE4D1 and PDE4D2 isoforms respectively cloned by PCR by Nemoz et al. [13]. The truncated human *PDE4D* cDNA isolated by Baecker et al. [36] could represent any of our pPDE43, pPDE39 or pPDE79 cDNAs. As the pPDE79 cDNA encodes a novel PDE4D isoform,

we will refer to it as PDE4D5, using the nomenclature of Beavo [2].

Alternative splicing of human *PDE4D* mRNAs

The structure of the human *PDE4D* mRNAs we have isolated is consistent with a complex pattern of alternative splicing (Figures 1 and 2). Two major points of alternative splicing are apparent. The first splice point occurs where the pPDE39, pPDE43 and pPDE79 mRNAs converge (A in Figure 1). As a result, all three of these mRNAs encode complete UCR1 and UCR2 (Figures 1 and 2). UCR1 and UCR2 are distinct regions of N-terminal sequence that are unique to the cAMP-specific PDEs, and are highly conserved among species as evolutionarily divergent as *D. melanogaster* and humans [12]. The point of divergence between the pPDE39, pPDE43 and pPDE79 mRNAs corresponds to the major point of alternative splicing in the *D. melanogaster dunce* mRNAs [5], and also in alternatively spliced mRNAs from the human *PDE4A* gene [12]. A second splice point occurs where the pPDE82 and pDun411 mRNAs diverge from the consensus (B in Figure 1). As a result, the pPDE82 and pDun411 mRNAs lack UCR1. This point of alternative splicing is not seen in transcripts from the *D. melanogaster dunce* gene, but is seen in alternatively spliced mRNAs from the human *PDE4B* gene [12].

In addition to the two major alternative splice points noted above, the pDun411 (PDE4D1) and pPDE82 (PDE4D2) mRNAs diverge from each other by the use of an additional alternative splice site. This observation is consistent with those made previously by others [13], and is also seen in the corresponding transcripts from the rat [7–9]. In brief, the PDE4D2 transcript is missing one or more alternatively spliced exon(s) that are present within the 5'-region of pPDE4D1 (indicated by the dashed line in Figure 1). The removal of these exon(s) from the PDE4D2 mRNA produces a change in the reading frame, so that initiation of translation at the PDE4D1 start codon does not produce a functional PDE. Translation is initiated in PDE4D2 at a downstream start codon, located in the middle of UCR2 (Figures 1 and 2).

Expression of the *PDE4D* cDNAs in COS cells

The vast majority of native cells that have been examined to date contain mixtures of PDE4 isoforms, which does not allow comparison of the properties of individual species [38]. To provide a single system that allows comparison of individual PDE4 isoforms, cDNAs encoding each of the five PDE4D isoforms were transiently expressed in COS cells under the control of the cytomegalovirus intermediate early gene promoter. Extracts from COS cells expressing each of the cDNAs were analysed by SDS/PAGE, followed by immunoblotting with either monoclonal or polyclonal antibodies designed to detect their common C-terminal region (see the Experimental section).

Each of the five recombinant proteins migrated in SDS/PAGE as single distinct species (Figure 3, top). The immunoreactive species detected by the PDE4D monoclonal antibody could be competed out by the immunogen (Figure 3, middle, gels a and b). In some, but not all instances, additional faint immunoreactive species were detected. However, these were not competed out by the immunogen and thus were likely to reflect non-specific interactions. The strong immunoreactive species detected by the monoclonal antibody was only seen in transfected COS cells and not in untransfected cells or those that had been mock-transfected (i.e. with the pcDNA3 plasmid containing no insert). This indicates that the species detected on the immunoblots indeed reflect expression of novel PDE4D forms of the sizes indicated.

Further evidence to support the identity of the bands seen on

Figure 3 Immunoblotting of recombinant and native PDE4D proteins

Top, Expression and subcellular distribution of PDE4D proteins in COS cells. COS cells were transfected with expression plasmids containing inserts from the indicated cDNAs. Homogenates from COS cells expressing these plasmids were fractionated into a low-speed pellet (P1), a high-speed pellet (P2), and high-speed supernatant, as described in the Experimental section. A 10 μ g amount of protein from each fraction was subjected to SDS/PAGE, and then immunoblotted with the 61D10E antibody. The positions of proteins used as size markers are indicated. All determinations of apparent molecular mass under denaturing conditions were performed on a minimum of three independent experiments, using unstained molecular-mass markers, as described previously [24]. Cells transfected with vector alone produced no detectable signal on immunoblotting. Middle, Extracts of COS cells transfected with plasmids encoding the indicated PDE4D species or control vector only (track c in all panels) were subjected to SDS/PAGE before immunoblotting with either the untreated PDE4D-specific 61D10E monoclonal antibody (gel a) or the monoclonal antibody that had been pretreated with the PDE4D immunogen GST-CT(1-66)4D (gel b). Treatment of the monoclonal antibody with either native GST or the shorter GST fusion protein GST-CT(1-40)4D failed to block recognition of the indicated major immunoreactive species (results not shown). Also shown (gel c) is an immunoblot of extracts of COS cells that had been transfected with VSV-epitope-tagged PDE4D3 (tracks 2 and 4) or mock-transfected cells (tracks 1 and 3) and then immunoblotted with either a PDE4D-specific polyclonal antiserum (tracks 1 and 2) or a VSV-specific monoclonal antibody (tracks 3 and 4). In both instances an identical immunoreactive species of 63 ± 3 kDa was identified. No such species was observed if no primary antibody was employed (results not shown). Bottom, Linearity of detection of various PDE4D proteins by the 61D10E antibody. Increasing concentrations of protein (2.25–10 μ g) from COS cells, transfected with a plasmid

Table 2 Subcellular distribution of human PDE4D proteins, as expressed in COS cells

Homogenates from COS cells expressing plasmids encoding various isoforms were fractionated into P1, P2 and supernatant, as described in the Experimental section. A 10 μ g amount of protein from each fraction was subjected to SDS/PAGE, and the relative amount of PDE protein in each lane was determined by immunoblotting. Each value is the mean $\pm$ S.D. for three separate experiments, each using separate transfections.

Isoform	P1 (%)	P2 (%)	Supernatant (%)
PDE4D1	4 $\pm$ 2	3 $\pm$ 1	93 $\pm$ 3
PDE4D2	2 $\pm$ 2	4 $\pm$ 2	94 $\pm$ 6
PDE4D3	11 $\pm$ 3	20 $\pm$ 2	69 $\pm$ 6
PDE4D4	20 $\pm$ 4	40 $\pm$ 7	40 $\pm$ 3
PDE4D5	18 $\pm$ 3	15 $\pm$ 1	67 $\pm$ 9

our immunoblots was obtained with the use of a VSV-epitope-tagged PDE4D3 species (see the Experimental section). Immunoblotting of COS cells expressing VSV-tagged PDE4D3 demonstrated the presence of a single co-migrating band (96 ± 3 kDa), using either a monoclonal antibody specific for the VSV antigen or a polyclonal antibody specific for PDE4D (Figure 3 middle, gel c). This band was indistinguishable in size from untagged recombinant PDE4D3. Thus transfection of COS cells with a plasmid encoding PDE4D3 has been shown by multiple criteria to produce a single species of around 96 kDa on SDS/PAGE.

The apparent molecular masses of the five recombinant PDE4D isoforms, as measured by SDS/PAGE, differed from that predicted from their amino acid sequence in all but one case, that of PDE4D1 (Table 1). It is possible that this may reflect particular conformational properties, or the anomalous binding to SDS, of these proteins in SDS/PAGE. Similar anomalous migration in SDS/PAGE has been observed for recombinant rat and human PDE4A proteins [21,26,35]. It is intriguing that PDE4D2 migrates anomalously, whereas PDE4D1 migrates as predicted, as PDE4D2 differs from PDE4D1 only by the deletion of amino acids 1–78 of PDE4D1. This region of PDE4D1 may produce a conformational change in the protein that affects its migration on SDS/PAGE.

We also demonstrated that we could use our monoclonal antibody to quantify relative amounts of the PDE4D forms (Figure 3, bottom). In these experiments, we used immunochemical detection as described in the Experimental section.

Partitioning of recombinant PDE4D proteins between the particulate fraction and the cytosol

To determine the relative intracellular distribution of the five human PDE4D proteins in COS cells expressing their respective plasmids, extracts of these cells were fractionated into a low-speed pellet (P1), a high-speed pellet (P2, i.e. the particulate fraction, which includes membranes) and a high-speed supernatant (i.e. cytosol). These fractions were then analysed by immunoblotting (Table 2). The PDE4D1 and PDE4D2 species partitioned exclusively into the high-speed supernatant, demonstrating that these proteins were located exclusively in the

expressing one of the PDE4D isoforms, were analysed by immunoblotting, using immunochemical detection as described for the determination of relative V_{max} values (see the Experimental section). The blots were scanned, and the intensity of the signal was plotted for each plasmid.

Figure 4 Analysis of native PDE4D isoforms

Top, Immunoblotting of unfractionated PDE4D proteins. Extracts from human cell lines or rat tissues were separated by SDS/PAGE and immunoblotted with the 61D10E antibody. Lanes are as follows: A, human HeLa cells; B, human SK-N-SH cells; C, rat brain. The positions of size markers are indicated. Bottom, analysis of PDE4D species in rat brain. (a) Immunoblot of PDE4D isoforms in fractions from rat brain. A rat brain homogenate was resolved into low-speed pellet (P1), high-speed pellet (P2) and high-speed supernatant (S) fractions (see the Experimental section). These fractions were subjected to SDS/PAGE and subsequent immunoblotting with the PDE4D-specific 61D10E antibody. Four immunoreactive species were noted. In the S fraction, in increasing size order, these corresponded to PDE4D1/2, PDE4D3, PDE4D5 and PDE4D4. The PDE4D1/2 band was not seen in the P1 or P2 fractions. (b) Analysis of PDE4D1 and PDE4D2 in rat brain by RT-PCR. Rat brain RNA was probed with primers specific for PDE4D1 (D1), which identified a sequence 369 bp in size. It was also analysed with primers (D1/2) able to detect both a PDE4D1 species (340 bp) and the PDE4D1 species (426 bp). A control lane (ctr), in which the samples were first treated with RNase A, is also shown.

cytosol. In contrast, the PDE4D3, PDE4D4 and PDE4D5 proteins appeared to partition between both the particulate and cytosolic fractions. However, there were profound differences between these three isoforms in the proportion found in the particulate fraction, ranging from approx. 30 % for PDE4D3 and PDE4D5 to 60 % for PDE4D4. In addition, the three species differed in their distribution between P1 and P2, with PDE4D5 present in a higher relative amount in P1, compared with PDE4D3 and PDE4D4 (Table 2). These differences in subcellular partitioning between the PDE4D3, PDE4D4 and PDE4D5 proteins were remarkably consistent for each isoform, even between different transfections.

Analysis of PDE4D4 isoforms occurring naturally in cells and tissues

We wished to demonstrate that the apparent molecular mass under denaturing conditions and cellular partitioning of the immunoreactive species detected in COS cells transfected with PDE4D cDNAs corresponded to that of species seen naturally in tissues and cells. For this purpose, we immunoblotted extracts from various human cell lines with our monoclonal antibody. Immunoreactive species of molecular mass indistinguishable from the recombinant PDE4D1/2, PDE4D3 and PDE4D5 isoforms were detected in HeLa cells (Figure 4, top). This is

Figure 5 Expression of the pPDE79 mRNA in human cell lines

RNase-protection assays with a probe corresponding to the alternatively spliced region of pPDE79 were performed using total RNA from various cell lines. The arrowhead demonstrates the position of the RNA fragment protected by the probe. SM refers to the size markers, representing *MspI*-digested pBR322, and the sizes of selected bands are indicated in nucleotides. Lanes are as follows: 1, position of probe without RNase; 2, the retinoblastoma cell line Y79; 3, the neuroblastoma cell line SK-N-SH; 4, the small cell lung cancer line Calu 3; 5, the embryonic tumour cell line Cates 1B; 6, the T-cell line Molt 3; 7, the B-cell Jurkat line; 8, HeLa S3 cells; 9, tRNA (10 µg).

consistent with our isolation of cDNAs encoding the PDE4D2 and PDE4D5 isoforms from a HeLa-cell cDNA library. We also detected an immunoreactive species corresponding to PDE4D5 in the neuroblastoma cell line SK-N-SH. In order to provide additional evidence that the 105 kDa band seen on immunoblots of the HeLa and SK-N-SH cell lines in fact represented the PDE4D5 protein, the presence of PDE4D5 mRNA in a variety of human cell lines was studied by RNase protection (Figure 5). An antisense riboprobe specific to the alternatively spliced region of the pPDE79 (PDE4D5) mRNA detected high levels of transcripts in the HeLa cell line, with clear but weaker signals in Calu 3 and SK-N-SH cells and no detectable signal in several other lines. Although these studies were performed on established tumour cell lines, they suggest that the relative abundance of the PDE4D5 mRNA may vary in different human cell types. Our immunoblot data are consistent with the previous detection of PDE4D1 and PDE4D2 as 68 kDa species in human mononuclear cells and the human Mono Mac 6 cell line [13,14].

To expand and confirm the data obtained from human cells, we examined tissues from the rat. Previously, we and others had demonstrated the presence of PDE4D mRNA protein in various rat tissues, but the relative contributions of the five different isoforms to the signals seen in those tissues was not determined [7,9,11,16,39,40]. Immunoblotting of rat brain detected species that migrated at the same size as human PDE4D3, PDE4D4 and PDE4D5 (Figure 4, bottom, gel a). The presence of a 93–96 kDa band consistent with PDE4D3 has been detected previously in other tissues from the rat [8,15,41,42]. In addition, we also noted on rat brain immunoblots a species of 68 kDa, which could represent PDE4D1, PDE4D2 or both [39]. To determine the identity of this 68 kDa species, we analysed rat brain for PDE4D1 and PDE4D2 mRNA by RT-PCR. This demonstrated that

Table 3 Solubilization of membrane-associated human PDE4D proteins by detergents or high salt concentrations

The high-speed pellet (P2) fraction was isolated from COS cells expressing the indicated cDNAs, and treated with NaCl or Triton X-100, or both. The preparations were then centrifuged as described in the Experimental section, and the amount of PDE4D protein in the resulting high-speed pellet (P2) fractions was measured by immunoblotting. Wash buffer (KHEM) was used as a negative control. Values are expressed as means $\pm$ S.D. for three separate experiments.

Isoform	Amount solubilized from particulate fraction (%)			
	Buffer only	1M NaCl	5% Triton X-100	NaCl + Triton X-100
PDE4D3	5 $\pm$ 3	13 $\pm$ 6	39 $\pm$ 8	93 $\pm$ 6
PDE4D4	6 $\pm$ 4	9 $\pm$ 7	12 $\pm$ 9	86 $\pm$ 11
PDE4D5	2 $\pm$ 3	42 $\pm$ 7	47 $\pm$ 8	94 $\pm$ 5

transcripts for both of the PDE4D1 and PDE4D2 species were present in rat brain (Figure 4, bottom, gel g).

Finally, we wished to confirm whether the subcellular distribution of the human PDE4D isoforms, as expressed in COS cells, was also representative of PDE4D isoforms present naturally in tissues. For this purpose, we prepared homogenates from rat brain and fractionated them in a manner identical with that used for our COS cell extracts. Immunoblotting of these fractions demonstrated that the PDE4D3, PDE4D4 and PDE4D5 proteins were present in both P2 and cytosolic fractions. However, the 68 kDa species in rat brain was seen exclusively in the cytosolic fraction (Figure 4, bottom, gel a). This could represent either or both PDE4D1 and PDE4D2, which migrate identically on SDS/PAGE (Table 1, Figure 3), and both of which were seen in RT-PCR analysis of rat brain.

Solubilization of particulate PDE4D3, PDE4D4 and PDE4D5

We have previously analysed the solubilization of particulate COS-cell-expressed rat PDE4A proteins. This was performed using the non-ionic detergent Triton X-100 to liberate integral membrane proteins and high-ionic strength solutions to liberate peripheral membrane proteins [24]. For example, we showed that the rat PDE4A1 protein can be released from the particulate fraction by treatment with the non-ionic detergent Triton X-100 [24,35], whereas the rat PDE4A5 and PDE4A8 proteins cannot be released from this fraction by treatment with either Triton X-

100 or high salt concentrations, or by a combination of both treatments [21,24,35,43]. Therefore, as part of the present study, we tested whether the three particulate-associated human PDE4D proteins, as expressed in COS cells, could be released from the high-speed particulate (P2) fraction by these treatments. The high-speed pellet fraction from COS cells expressing one of the PDE4D3, PDE4D4 and PDE4D5 plasmids was treated with various concentrations of either Triton X-100 or NaCl, or both, and the release of PDE protein from the pellet fraction was analysed by immunoblotting (Table 3). PDE4D3 could be partly solubilized by Triton X-100 alone but not by high concentrations of NaCl, whereas PDE4D4 was resistant to both treatments and PDE4D5 could be partly solubilized by either of these two treatments. However, the particulate forms of all three proteins could be completely solubilized by Triton X-100 and NaCl when used together. We discuss the potential physiological implications of these findings below (see the Discussion section).

The PDE4D1, PDE4D2, PDE4D3, PDE4D4 and PDE4D5 cDNAs all encode high-affinity cAMP PDEs

The kinetic properties of the five human PDE4D enzymes were measured in preparations from COS cells expressing their respective cDNAs. The average PDE activity in mock-transfected (vector only) COS cells was 10 ± 11 pmol of cAMP hydrolysed/min per mg of protein ($n = 6$ separate transfections; mean $\pm$ S.D.). In COS cells transfected with a plasmid expressing PDE4D1, this rose to 298 ± 19 pmol of cAMP hydrolysed/min per mg of protein. This was typical of transfections for all five PDE4D isoforms, in that endogenous PDE4D activity was less than 4% of activity after transfection. The K_m for cAMP for the various PDE4D isoforms was approx. 1 mM (Table 4), which is in the range typical for PDE4D isoforms [1–3].

Determination of relative V_{max} values for the PDE4D isoforms

We wished to determine whether the various PDE4D isoforms, as expressed in COS cells, had different maximal catalytic activities (V_{max} values). To do this we first determined maximal catalytic activity by substrate-utilization analysis and fitting to the non-linear form of the Michaelis–Menten equation (see the Experimental section). However, as the absolute amount of each of the PDE4D proteins is not known in each of the fractions, we

Table 4 K_m for cAMP, IC_{50} for rolipram, Hill coefficients and relative V_{max} values for the human PDE4D isoenzymes

The properties of the isoforms were determined by expressing their cDNAs in COS cells. High-speed pellet (P2) and supernatant (cytosolic) fractions were isolated from COS cells expressing the indicated cDNAs. K_m values (in μ M) were determined over the substrate range 0.25–25 μ M cAMP (seven different concentrations), and were obtained by non-linear fitting of data to the Michaelis equation. IC_{50} values (in μ M) for rolipram inhibition were obtained with 1 μ M cAMP as substrate, and with eight different concentrations of inhibitor. Relative V_{max} values were determined from Lineweaver–Burk plots, with quantitative immunoblotting performed (see the Experimental section) to determine the relative amounts of the PDE4D protein in the assays. Relative V_{max} values for the cytosolic activities are given relative to those of the PDE4D1 protein, which is set as 1.0. Relative V_{max} values for the particulate activities are given relative to the corresponding cytosolic species for each particular isoform. Values are given as means $\pm$ S.D. for three independent experiments (six for PDE4D3), each using separate transfections. n/a, not applicable.

Isoform	K_m		IC_{50}		Cytosolic V_{max} (relative to PDE4D1)	Particulate V_{max}	Hill coefficients	
	Cytosolic	Particulate	Cytosolic	Particulate			Cytosolic	Particulate
PDE4D1	1.2 $\pm$ 0.3	n/a	0.05 $\pm$ 0.01	n/a	(1)	n/a	0.47 $\pm$ 0.12	n/a
PDE4D2	1.3 $\pm$ 0.2	n/a	0.05 $\pm$ 0.01	n/a	1.1 $\pm$ 0.2	n/a	0.61 $\pm$ 0.05	n/a
PDE4D3	1.4 $\pm$ 0.3	0.6 $\pm$ 0.2	0.14 $\pm$ 0.01	0.32 $\pm$ 0.05	1.2 $\pm$ 0.3	0.91	0.93 $\pm$ 0.08	0.94 $\pm$ 0.03
PDE4D4	1.1 $\pm$ 0.1	0.8 $\pm$ 0.1	0.06 $\pm$ 0.01	0.05 $\pm$ 0.02	2.9 $\pm$ 0.3	2.5	0.50 $\pm$ 0.05	0.35 $\pm$ 0.08
PDE4D5	1.0 $\pm$ 0.2	0.8 $\pm$ 0.2	0.08 $\pm$ 0.01	0.59 $\pm$ 0.05	1.5 $\pm$ 0.2	3.3	0.54 $\pm$ 0.05	0.75 $\pm$ 0.09

Figure 6 Dose-response curves for the inhibition of the PDE4D enzymes by rolipram

Top, Comparison of the five cytosolic forms, middle, comparison of the three particulate forms; bottom, comparison of the cytosolic and particulate forms of PDE4D3. Values were determined as in the legend to Table 4.

determined relative $V_{\max}$ activities by using a qualitative immunoblotting approach (Figure 3, bottom panel), to identify the relative levels of expression of each of the isoforms in both particulate and cytosolic fractions. We thus determined the $V_{\max}$ activity for each of the cytosolic components relative to that of PDE4D1 (Table 4). To determine whether the activities of the particulate-associated enzymes were different from the cytosolic enzymes, we determined each of their activities relative to that of their corresponding cytosolic forms (Table 4). This analysis is impossible to achieve by other means, as purification of a particulate enzyme to homogeneity would necessitate its removal from the particulate fraction. Although the relative $V_{\max}$ value does not give an absolute figure for catalytic activity, it does allow us to determine whether the various PDE4D species have different activities when expressed in COS cells and whether the particulate-associated forms have activities that differ from their cytosolic equivalent.

Four of the five cytosolic forms had very similar relative $V_{\max}$ values (Table 4). However, the cytosolic form of PDE4D4 had a relative $V_{\max}$ that was 2–3-fold higher than that of the other four cytosolic enzymes ($P < 0.001$). Comparison of the relative $V_{\max}$ of the cytosolic and particulate forms of PDE4D3 showed them to be very similar. However, the relative $V_{\max}$ of the particulate forms of PDE4D4 and PDE4D5 were both approx. 3-fold higher than their corresponding cytosolic forms ($P < 0.001$, Table 4).

Inhibition of recombinant PDE4D enzymes by rolipram

Dose-dependent inhibition of the COS-cell-expressed human PDE4D enzymes by rolipram generated values (Table 4) typical for members of the PDE4 family [1–3]. High concentrations of rolipram almost completely abolished detectable PDE activity in the extracts, consistent with the expressed enzyme being by far the major PDE activity in the transfected cells. IC_{50} values of the various cytosolic forms varied between 0.05 and 0.14 μM (Table 4). IC_{50} values of the particulate forms of PDE4D3 and PDE4D5 were 2–7-fold higher (0.32 and 0.59 μM) than those of their respective cytosolic forms ($P < 0.01$ and $P < 0.001$ respectively; Table 4). These data suggest that association with the particulate fraction may change the conformation of PDE4D3 and PDE4D5 and hence their susceptibility to inhibition by rolipram, as discussed in detail below (see the Discussion section).

Dose-response curves for rolipram

Previous analysis of the inhibition of various human PDE4 isoforms by rolipram has demonstrated that the dose-response curves deviate from those expected for simple Michaelian kinetics [12,26,44–46]. Therefore we analysed the dose-response curves for the inhibition of our human PDE4D isoforms by rolipram (Figure 6) and derived Hill coefficients from these curves (Table 4). All the isoforms, except for PDE4D3, deviated from simple Michaelian kinetics. The inhibitor curves for the cytosolic forms of the five proteins were quite similar, except for PDE4D3, which had a much steeper slope (Figure 6; top, Table 4). Similarly, the curves for the particulate forms of PDE4D3 and PDE4D5 had a steeper slope than that of the particulate form of PDE4D4 (Figure 6, middle). The association of PDE4D3, PDE4D4 and PDE4D5 with the particulate fraction produced a modest (less than 2-fold) difference in slope (Figure 6, bottom; Table 4), despite the changes in IC_{50} for PDE4D3 and PDE4D5. Our data demonstrate that only the cytosolic and particulate forms of PDE4D3 showed simple Michaelian kinetics, with a Hill coefficient approaching unity, and that the other forms demonstrated

apparent negative co-operativity. This apparently negative co-operativity could occur as a result of several possible mechanisms, including subunit interactions, multiple rolipram-binding sites or the presence of multiple kinetic forms.

Our data demonstrate that the various PDE4D isoforms differ in their ability to be inhibited by rolipram, yet have no detectable difference in K_m for cAMP. This suggests that the amino acids involved in rolipram binding may not be exactly the same as those involved in the binding of cAMP. This possibility has been suggested previously by three lines of evidence. (i) Single point mutations in a PDE4B species can completely block rolipram inhibition while having little effect on K_m for cAMP or relative V_{max} [47]. (ii) The *D. melanogaster dunce* PDE, which serves as a paradigm for the PDE4 enzyme family, is not inhibited by rolipram, despite having UCR1 and UCR2, a similar sequence (85% amino acid sequence identity) for the catalytic site and a similar K_m for cAMP, when compared with the mammalian PDE4s [12]. (iii) Particulate association of PDE4A5 leads to a profound increase in sensitivity to rolipram inhibition with no changes in K_m for cAMP and little change in relative V_{max} [26]. Thus rolipram inhibition can serve as a detector of changes in PDE4 conformation, which may or may not be associated with changes in K_m or relative V_{max} .

DISCUSSION

In this study, we describe the complete structure of cDNAs encoding two novel different human cAMP-specific PDE isoforms, PDE4D4 and PDE4D5. The structure of these two cDNAs, and those of the three human PDE4D isoforms reported previously [12,13,36], is consistent with alternative splicing of their corresponding mRNA transcripts, as well as initiation of transcription at multiple sites within the *PDE4D* gene. As a starting point to understanding the role of these individual forms in an organism, we wished to express all five isoforms in a common system, which would provide a direct comparison of their properties.

Although study of the structure of the human *PDE4D* gene will be necessary to confirm that our clones are indeed generated by alternative mRNA splicing, we believe that it is unlikely that any of our cDNA clones are products of artifacts of cloning for three reasons. First, our PDE4D1, PDE4D2, PDE4D3 and PDE4D5 cDNAs, when expressed in COS cells, each generate a protein with an apparent molecular mass under denaturing conditions that is very similar to that of PDE4D species occurring naturally in human cell lines. Secondly, the apparent molecular masses of all five recombinant human proteins are similar to those of native isoforms in the rat. Thirdly, there is impressive (greater than 90% identity) conservation of amino acid sequence encoded by cDNAs between the corresponding rat and human PDE4D1, PDE4D2 and PDE4D3 isoforms [7–9,11–13], which provides additional confirmation of the structures of these cDNAs. Our immunoblot data also suggest that there is close conservation between the human and rat PDE4D4 and PDE4D5 isoforms, although we have yet to isolate cDNAs for the rat isoforms. It is not certain if the five species we have cloned and see on immunoblots reflect all possible PDE4D isoforms. However, immunoblotting of various cell lines and brain by ourselves and others [9,13,15,39] with PDE4D-specific antisera has not indicated the presence of immunoreactive species that migrate differently from those detected in this study.

Our data demonstrate that the structure of the mammalian *PDE4D* gene is much more complicated than the partial rat genomic structure that has been reported previously [16]. That study mapped only exons present in the PDE4D1 and PDE4D2

isoforms, which are splice variants that lack UCR1 [12] (Figures 1 and 2). As we have now isolated three cDNAs that contain UCR1 (i.e. PDE4D3, pPDE4D4 and PDE4D5), it is likely that a substantial number of 5'-exons are missing from this genomic map.

Previously, we reported the biochemical properties of a truncated form of human PDE4D, expressed in the yeast *Saccharomyces cerevisiae* [12]. The protein we analysed in those studies was encoded by an engineered construct designed to initiate translation at the phenylalanine at the N-terminus of the sequences in common to PDE4D3, PDE4D4 and PDE4D5 (i.e. at splice point A; Figures 1 and 2). The properties of a different human PDE4D N-terminal truncation have also been reported [36]. Nemoz et al. [13] have expressed human PDE4D2 and PDE4D3 in 293 cells, but no inhibitor, kinetic or cell-localization analysis was reported [13].

Our data provide novel insight into the functions of the N-terminal regions of the PDE4D proteins. Three of the human PDE4D isoforms (PDE4D3, PDE4D4 and PDE4D5) contain both UCR1 and UCR2 and could be called the 'long' isoforms. The other two (PDE4D1 and PDE4D2) lack UCR1 and could be called the 'short' isoforms. Although the biochemical properties of each of the 'long' forms is different, the most striking difference between all the 'long' forms and the two 'short' forms is that only the 'long' forms have a portion of the enzyme activity that is present in the particulate fraction. This particulate fraction of the 'long' forms can only be solubilized by detergent and at high ionic strength, implying a specific interaction between the PDE and a membrane component. The nature of this membrane component is not known, but it could be a specific 'anchoring' protein, a cytoskeletal element or a component of a signalling pathway. The differences in solubilization among the three particulate-associated 'long' forms (Table 3) suggest that the mechanism of association may differ between them. Although the exact mechanism for association remains unclear, our data strongly suggest that an important function of the N-terminal regions is to mediate the subcellular localization of the 'long' forms of the PDEs.

Our localization data use expression of the PDE4D proteins in COS cells as a model system for studying their enzymology and intracellular location. It is possible that membrane structures or other cell components normally required for targeting of the PDE4D isoforms (e.g. 'anchoring' proteins) may be absent from or qualitatively different in COS cells, as compared with cells that naturally contain these isoforms. Overexpression of the proteins in cells may also affect their intracellular distribution. However, at a minimum, our data indicate a propensity for the 'long' PDE isoforms to interact with the particulate fraction of the cell. This conclusion is additionally supported by our analyses of 'long' forms present natively in brain, where they are found in the particulate as well as the cytosolic fraction. Our previous studies on the rat PDE4A proteins demonstrated similar subcellular localization data for the proteins expressed in COS cells as compared with the corresponding native proteins in the brain [21,24,35].

Our data also support the concept that association with the particulate fraction can influence the enzymic properties of the PDE4D isoforms. For example, a 7-fold change in the IC_{50} for rolipram and a 3-fold change in relative V_{max} was seen between the particulate and cytosolic forms of PDE4D5 (Table 4). These data indicate that the particulate-associated isoforms can adopt a conformation that is distinct from that of their corresponding cytosolic isoforms. This association with the particulate fraction may change the conformation of these enzymes and hence their susceptibility to inhibition by rolipram. Similar effects have been

noted for the PDE4A isoforms, especially human PDE4A4B expressed in COS cells [26].

The existence of multiple human PDE4D proteins, each having a catalytic region with identical amino acid sequence, potentially complicates the selective use of pharmacological agents that interact only with the catalytic region of the isoforms. One alternative approach would be to develop reagents targeted to the alternatively spliced regions of the mRNAs, perhaps by the use of antisense strategies. Agents that could be targeted to the N-terminal regions of the proteins might allow modulation of their intracellular localization. Such agents would modulate not only the localization of the isoforms, but also their conformation and kinetic properties.

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