

# Expression, intracellular distribution and basis for lack of catalytic activity of the PDE4A7 isoform encoded by the human *PDE4A* cAMP-specific phosphodiesterase gene

Lee Ann JOHNSTON<sup>\*1</sup>, Suat ERDOGAN<sup>\*2</sup>, York Fong CHEUNG<sup>\*</sup>, Michael SULLIVAN<sup>\*3</sup>, Rachael BARBER<sup>†</sup>, Martin J. LYNCH<sup>\*</sup>, George S. BAILLIE<sup>\*</sup>, Gino VAN HEEKE<sup>‡</sup>, David R. ADAMS<sup>‡</sup>, Elaine HUSTON<sup>\*</sup> and Miles D. HOUSLAY<sup>\*4</sup>

<sup>\*</sup>Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Wolfson Building, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., <sup>†</sup>Respiratory Disease Area, Novartis Institutes for BioMedical Research, Wimblehurst Road, Horsham RH12 5AB, U.K., and <sup>‡</sup>Department of Chemistry, Heriot-Watt University, Riccarton Campus, Edinburgh EH14 4AS, Scotland, U.K.

PDE4A7 is an isoform encoded by the human *PDE4A* cAMP-specific phosphodiesterase gene that fails to hydrolyse cAMP and whose transcripts are widely expressed. Removal of either the N- or C-terminal unique portions of PDE4A7 did not reconstitute catalytic activity, showing that they did not exert a chronic inhibitory effect. A chimera (Hyb2), formed by swapping the unique N-terminal portion of PDE4A7 with that of the active PDE4A4C form, was not catalytically active. However, one formed (Hyb1) by swapping the unique C-terminal portion of PDE4A7 with that common to all active PDE4 isoforms was catalytically active. Compared with the active PDE4A4B isoform, Hyb1 exhibited a similar  $K_m$  value for cAMP and  $IC_{50}$  value for rolipram inhibition, but was less sensitive to inhibition by Ro-20-1724 and denbufylline, and considerably more sensitive to thermal denaturation. The unique C-terminal region of PDE4A7 was unable to support an active catalytic unit, whereas its unique N-terminal

region can. The N-terminal portion of the PDE4 catalytic unit is essential for catalytic activity and can be supplied by either highly conserved sequence found in active PDE4 isoforms from all four PDE4 subfamilies or the unique N-terminal portion of PDE4A7. A discrete portion of the conserved C-terminal region in active PDE4A isoforms underpins their aberrant migration on SDS/PAGE. Unlike active PDE4A isoforms, PDE4A7 is exclusively localized to the P1 particulate fraction in cells. A region located within the C-terminal portion of active PDE4 isoforms prevents such exclusive targeting. Three functional regions in PDE4A isoforms are identified, which influence catalytic activity, subcellular targeting and conformational status.

**Key words:** cAMP-specific phosphodiesterase (PDE4A7), catalytically inactive isoform, intracellular targeting, phosphodiesterase, rolipram.

## INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDEs) provide the sole means of inactivating the second messengers cAMP and cGMP within cells and thus are poised to play key roles in regulating many major signalling processes [1–5]. Of the 12 known PDE families, nine are able to hydrolyse cAMP to 5'-AMP. Each family comprises a number of isoforms that can arise from multiple genes, alternative mRNA splicing and the use of distinct promoters. Thus cells can tailor their regulation of cAMP signalling by altering the pattern of expression of their PDE isoforms. These various PDE isoforms are characterized by having distinct patterns of intracellular distribution, as well as particular kinetic and regulatory properties, which allow for their regulation by other signalling pathways. Differences in the distribution of PDEs have been exploited therapeutically, with inhibitors selective for PDE3 being used to treat intermittent claudication [6,7], for PDE5 to treat erectile dysfunction [8–10] and, more recently, for PDE4 to treat chronic obstructive pulmonary disease and asthma [11–17].

PDE4 enzymes specifically hydrolyse cAMP, with the human family encoded by four genes [3,18–21]. Additional complexity in this family is generated by the use of multiple promoters coupled to alternative mRNA splicing, yielding isoforms with

unique N-terminal regions. In a number of PDE4 isoforms, an important role for the unique N-terminal region is in conferring intracellular targeting [5,20]. This may involve protein–protein interactions as shown, for example, in the binding of PDE4D5 to RACK1 (receptor for activated C-kinase 1) [22] and in the binding of PDE4A5 to the immunophilin XAP2 [23]. It can also involve lipid–protein interaction, as shown in the TAPAS (tryptophan anchoring phosphatidic acid selective-binding)-domain interaction with phosphatidic acid that was shown to determine the binding of PDE4A1 to bilayer membranes [24]. These N-terminal regions can also influence catalytic activity ( $V_{max}$ ) and the conformation of the catalytic unit, as determined by altered thermostability (for example, see [25,26]). Active PDE4 enzymes are also characterized by two highly conserved regions, called UCR1 and UCR2 (upstream conserved regions 1 and 2), which are located between the isoform-specific N-terminal region and the catalytic domain [19]. Alternative mRNA splicing then generates 'long' PDE4 isoforms, having UCR1 and UCR2, 'short' isoforms having only UCR2 and 'super-short' isoforms that have a truncated UCR2.

The human *PDE4A* gene has been characterized and localized to chromosome 19p–13.1 in man [27]. It generates at least three active isoforms, namely the PDE4A4B and PDE4A10 long

Abbreviations used: PBMC, peripheral blood mononuclear cell; PDE, cyclic nucleotide phosphodiesterase; PDE4, cAMP-specific phosphodiesterase; RT-PCR, reverse transcriptase PCR; UCR, upstream conserved region; VSV, vesicular-stomatitis virus.

<sup>1</sup> Present address: Randox Laboratories Ltd., 55 Diamond Road, Crumlin, County Antrim, BT29 4QY, Northern Ireland.

<sup>2</sup> Present address: Department of Biochemistry, Faculty of Veterinary Medicine, University of Mustafa Kemal, Tayfur Sokmen Campus, 31040 Antakya, Turkey.

<sup>3</sup> Present address: Astra Charnwood, Bakewell Road, Loughborough LE11 0RH, U.K.

<sup>4</sup> To whom correspondence should be addressed (e-mail M.Houslay@bio.gla.ac.uk).

isoforms and the PDE4A1 short isoform [19,26,27]. These, similar to active isoforms encoded by all *PDE4* genes, are generated solely by 5' splicing. However, the *PDE4A* gene also encodes a 324-residue isoform called PDE4A7 that, uniquely to date, arises from both 5' and 3' domain swaps. This gives PDE4A7 both a unique 32-residue N-terminal region and a unique 14-residue C-terminal region [27,28]. These isoform-specific regions are positioned around a core that is highly conserved amongst the PDE4 subfamilies and which contains the active site. In the present study we show that the *PDE4A* gene, similar to those for various protein kinases [29], can encode catalytically inactive isoforms. In determining the basis of this lack of PDE activity, we identified a number of functionally active regions in PDE4A isoforms that relate to the functioning of the catalytic unit, intracellular targeting and conformation.

## EXPERIMENTAL

### Materials

Restriction enzymes, Dulbecco's modified Eagle's medium, fetal calf serum were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). Tris, Hepes, DEAE-dextran (molecular mass cut-off 500 000), cytochalasin B, benzamidine hydrochloride, PMSF, aprotinin, pepstatin A, antipain, EDTA, EGTA, cAMP, cGMP, Dowex 1 × 8-400 (chloride form, 200–400 mesh), 3-isobutyl 1-methylxanthine, snake venom (*Ophiophagus hannah*), VSV (vesicular-stomatitis virus)-epitope antibody and bovine brain calmodulin were from Sigma (Poole, Dorset, U.K.). [<sup>3</sup>H]cAMP and [<sup>3</sup>H]cGMP were from Amersham International. Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates, London, U.K.). Dithiothreitol, Triton X-100, and Triton X-114 were from Boehringer (Lewes, East Sussex, U.K.). Triethanolamine was from BDH (Glasgow, Scotland, U.K.). Glycerol was from Fison (Loughborough, Leicestershire, U.K.). Bradford reagent was from Bio-Rad (Hemel Hempstead, Herts., U.K.). DMSO was from Koch-Light (Colnbrook, Bucks., U.K.). Rolipram {4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone} was generously provided by Schering Aktiengesellschaft (Berlin, Germany). The plasmid pBluescript-pde46 containing the PDE4A4B clone was generously provided by Dr Graeme Bolger (Comprehensive Cancer Center, University of Alabama, Birmingham, AL, U.S.A.). The antisera specific for the C-terminal region conserved in all active PDE4A species has been described previously [30].

### Human PDE4A4 clones

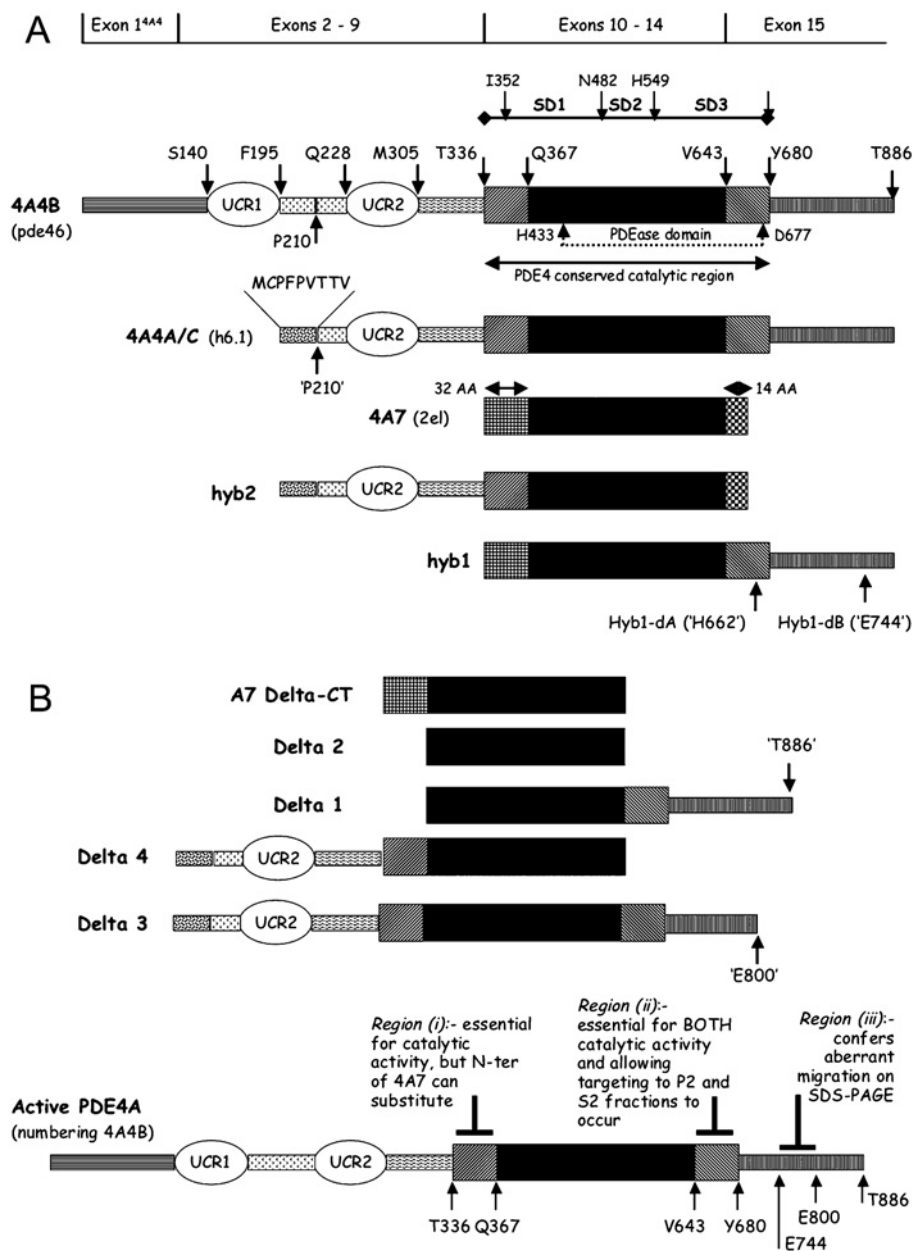
A number of human PDE4A clones have been described. A full-length species called PDE4A4B (pde-46) was reported by Bolger et al. [19], as well as two N-terminally truncated forms of this species, namely PDE4A4C (h6.1 h; PDE-IVA-h6.1) [31] and the first human PDE4 to be cloned, PDE4A4A (h-PDE1) [32]. PDE4A4C was generated [31] from two overlapping cDNA clones whose sequence encompassed a region, which is nearly identical to that found within PDE4A4B, and which runs from amino acid residue at position 210 of PDE4A4B to the extreme C-terminus of PDE4A4B (position 886) (Figure 1). It thus encompasses the entire putative catalytic region together with the C-terminal region of PDE4A4B, but lacks the N-terminal region of PDE4A4B. However, to generate an active construct, a PCR-based strategy was used to add an initiator methionine residue together with the N-terminal nine additional amino acid residues reported in PDE4A4A (h-PDE1), the first human PDE4 clone to be isolated [32]. This active construct was called PDE4A4C (h6.1), which

thus complemented PDE4A4A (h-PDE1). It should be noted, however, that subsequent analysis of the human PDE4A gene has shown that the unique nine amino acids and the apparent site of splicing in PDE4A4A (h-PDE1) must have arisen as a cloning artifact [27]. Thus PDE4A4B is the true isoform of these species, whereas PDE4A4A/C provide useful active truncates which lack the isoform-specific N-terminal region.

### Isolation of cells from peripheral blood and extraction of RNA for cDNA synthesis and RT (reverse transcriptase)-PCR

Blood samples were collected using either 50 ml falcon tubes containing 2 ml of filter sterilized 3.8% (w/v) trisodium citrate or using 10 ml heparin vacutainer tubes. PBMCs (peripheral blood mononuclear cells) were separated from granulocytes and erythrocytes using a Ficoll gradient, essentially according to the manufacturer's instructions. Briefly, the blood was diluted 1:1 with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS, and up to 25 ml of this mixture was layered carefully on to 15 ml Ficoll-Paque in a 50 ml falcon tube at 20 °C. A Ficoll gradient was established by centrifugation at 400 g for 30 min at 20 °C. The layer containing PBMCs was recovered and transferred to fresh ice-cold falcon tubes. The remaining serum and Ficoll was aspirated to leave the erythrocyte pellet. T-cells and monocytes were purified sequentially from the PBMC layer, while neutrophils were purified from the erythrocyte pellet, as described below. Blood cells in the recovered PBMC layer were washed twice with ice-cold MACS buffer (PBS, 0.4% BSA and 2 mM EDTA), the number of cells was counted, and resuspended in 80 µl of MACS buffer for every 10<sup>7</sup> cells present. To this, 20 µl of each CD4 and CD8 Multisort MACS beads were added and T-cells were separated using a MACS VS+ column according to the manufacturer's instructions. Monocytes were purified from the first flow-through fraction of this procedure using CD14 MACS microbeads. Neutrophils were isolated from the erythrocyte pellet generated by the Ficoll gradient. The cell pellet was drained and erythrocytes selectively lysed twice by resuspension in cold sterile water for exactly 30 s, followed immediately by the addition of an equal volume of 2 × PBS, before centrifugation. The remaining leucocytes were washed in 20 ml of MACS buffer, and resuspended in a final volume of 50 µl of MACS buffer for every 5 × 10<sup>7</sup> cells present. Neutrophils were recovered by adding an equal volume of CD16 MACS Microbeads followed by separation on a type C MACS column, as described by the manufacturer. In all cases, the recovered cells were counted, centrifuged at 400 g for 5 min at 4 °C and the supernatant fraction discarded. The recovered pellets were lysed in 60 µl of RLT buffer (RNA Lysis-Thiocyanate buffer, Qiagen) for every 10<sup>6</sup> cells present, but using not less than 350 µl. Samples were kept chilled at all times to minimize degradation. The lysate was thoroughly disrupted by pipetting up and down, and flash frozen at –70 °C.

Frozen lysates were warmed at 37 °C for 10 min, and homogenized using QIAshredder columns (Qiagen), according to the manufacturer's instructions. RNA was extracted from the homogenized lysates using QIAgen's RNeasy Mini or Midi Kits following the kit instructions. The RNA was treated with RQ1 DNase (1 units/µl) to remove genomic DNA contamination. RNA (75 µl) was mixed with 20 µl of RQ1 and 5 µl of 20 × RQ1 Buffer (0.6 mM Tris/HCl, 72 mM MgCl<sub>2</sub> and 120 mM NaCl), and incubated at 37 °C for 30–45 min. After this, the RNA was re-purified using the 'clean-up' protocol of the RNeasy Mini Kit. To check for successful genomic DNA removal, each sample was tested in a RT-PCR for β-actin with and without reverse transcriptase, using Promega's Access RT-PCR Kit. DNA-free samples were subjected to first-strand cDNA synthesis using



**Figure 1** Schematic of various PDE4A isoforms, truncates and chimeras

The key elements of the long PDE4A4B (pde46) isoform [19,30,36], including the regulatory UCR1 and UCR2 regions plus the core region (solid black) shared with PDE4A7, are shown. The region conserved amongst all PDE4 subfamilies is shown (PDE4 conserved catalytic region) and the extent of the PDEase domain identified in motif search programs. Also shown is the PDE4A4A/C active form and the PDE4A7 inactive form together with various chimeras and truncates generated in the present study. The boundaries of various regions/truncations are indicated with the amino acid numbering in terms of the known PDE4A4B sequence for comparison. These include the three subdomains namely SD1 (helix-0 to helix-7), SD2 (helix-8 to helix-11) and SD3 (helix-12 to helix-16) of the catalytic unit, as inferred from structural studies performed on PDE4B and PDE4D [46,47,71]. HSPDE4A7, 2EL (GenBank<sup>®</sup> accession no. U18088); HSPDE4A4B, pde-46 (GenBank<sup>®</sup> accession no. L20965); HSPDE4A4C, h6.1, hPDE-IVA-h6.1 (GenBank<sup>®</sup> accession no. U18087); HSPDE4A4A, hPDE-1 (GenBank<sup>®</sup> accession no. M37744).

the Pharmacia first-strand cDNA synthesis kit. RNA (5 µg) was diluted to 20 µl using nuclease-free water and then heated to 65 °C for 10 min. To this, 14 µl of a mix containing random primers, oligo d(T), buffer and enzyme (supplied with kit) was added. This was incubated for 1 h at 37 °C, followed by a 90 °C incubation for 5 min to inactivate the reverse transcriptase. The completed reaction was diluted with nuclease-free water to 1 ml, to allow addition of 5 µl (equivalent to the addition of 25 ng of RNA) for subsequent PCR.

First-strand cDNA was used as template for PCR analysis of PDE4A4B, PDE4A10 and PDE4A7. PCR primers were designed to cover the unique 5' end of the mRNA to target a specific splice variant. The sequences used were obtained from the GenBank<sup>®</sup> database, and GenBank<sup>®</sup> accession numbers are given below. Additional primers were designed to amplify β-actin and transferrin as amplification controls and for normalization purposes. Primers were designed using the Applied Biosystems software Primer Express, and selected for their position within

the sequence and calculated annealing temperature. Amplified PCR fragments were fractionated and visualized on a 2% agarose gel in 1× TAE buffer (40 mM Tris/acetate, 1 mM EDTA) containing 1 µg/ml ethidium bromide. Amplification conditions and primer sequences were tested and optimized on first-strand cDNA synthesized from total brain RNA (Clontech). Reaction conditions were optimized to enable detection in as little as 25 ng of total RNA. Brain RNA, similarly converted to cDNA, served as positive control for all PCR reactions, as all tested PDE variants were found to be present in brain tissue. PCR reactions were performed on each sample in duplicate or triplicate using the following conditions: 18.75 pmol of each forward and reverse primer; 12.5 µl of 2× PCR Mix; 5 µl of the cDNA synthesis reaction equivalent to 25 ng of RNA; nuclease-free water to a total volume of 25 µl. Amplifications were performed using either Epicentre's 2× PCR Mix H and 0.625 unit of Epicentre's MasterAmp Amplitherm DNA Polymerase for amplification of PDE4A4 or using Qiagen's HotStarTaq Mastermix for all other reactions, and subjected to the following program on a Biometra T3 Thermocycler: 95 °C for 5 min; 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; a final extension of 72 °C for 10 min; followed by a pause at 4 °C. Semi-quantitative RT-PCR was performed in duplicate by halting the PCR amplification during the exponential phase as determined for each variant in preliminary experiments. Preliminary experiments had shown that  $\beta$ -actin was a better and more constant standard for comparisons. PCR products were separated on a 2% agarose gel. Sequences of PCR primers used were: PDE4A4B (GenBank® accession no. L20965) forward, CGG AAA GGA GCC TGT CTC TG; PDE4A4B (GenBank® accession no. L20965) reverse, AGT GCC ATG GAA GGA CGA GG, to give 257 bp. PDE4A10 (GenBank® accession no. AF073745) forward, AGA TCT GTC AGC TTC GAG GCA G; PDE4A10 (GenBank® accession no. AF073745) reverse, AGT GAG AAG TTG CTA CGG ACG C, to give 281 bp. PDE4A7 (GenBank® accession no. U18088) forward, GTC CAA GCA CAT GAC CCT CCT; PDE4A7 (GenBank® accession no. AF073745) reverse, CCG AGC CTG TAC CTG AGA CTT, to give 301 bp.

### Generation of chimeras and truncates

To generate the chimera Hyb2 with a C-terminal VSV epitope tag the C-terminal encoding region of PDE4A7 was amplified from the plasmid p19-2EL [28] in a PCR reaction using primers pde4-pde1 and 2EL3'. Primer pde4-pde1 (5'-TCGGAGCTGGC-GCTCATG-3') anneals upstream of the unique *XhoI* site in PDEA4C, whereas primer 2EL3' (5'-ACCGGTATAAAACCC-ACCCTGT-3') anneals with the sequence encoding the C-terminus of PDE4A7 and contains an *AgeI* restriction site that replaces the PDE4A7 stop codon. The PCR product was subcloned into pCRscript (Stratagene) and sequenced to ensure that no sequence changes had occurred. One clone, which carried no amino acid encoding changes, was digested with *AgeI* and *HindIII* and ligated to annealed oligonucleotides VSVa (5'-CC-GGTCCTTACACCGATATAGAGATGAACAGGCTGGGAAA-GTGATCTAGA-3') and VSVb (5'-AGCTTCTAGATCACTTTC-CCAGCCTGTTTCATCTCTATATCGGTGTAAGGA-3'). These oligonucleotides encode the VSV epitope tag and contain a stop codon followed by an *XbaI* restriction site. Following sequence analysis, the epitope-tagged C-terminal encoding region was released as an *XhoI/XbaI* fragment and ligated with *XhoI/XbaI*-digested pCMV-6.1 to create the plasmid pCMV-HYB2vsv.

To generate PDE4A7 with a C-terminal VSV epitope tag, the N-terminal encoding region of the tagged Hyb2 in plasmid pCMV-HYB2vsv was removed on a *BamHI/XhoI* fragment and replaced

with the N-terminal encoding region of PDE4A7 from the plasmid p19-HYB1 (*BamHI/XhoI*) to create the plasmid pCMV-2ELvsv.

To generate Hyb1, the open reading frame of PDEA4C in p18-SKB5/6.1 was released by digestion with *BamHI* and *SalI* and ligated into a derivative of pUC19 devoid of the *EcoRI* site, p19E. The resulting plasmid, p19E-6.1, was digested with *BamHI* and *XhoI* to remove the 5' end of PDEA4C and replace it with the 5' end of PDE4A7, amplified from p19-2EL using the primers 2EL-5' (5'-AGCTGGATCCACCATGGTGCTTCCTTCAGACC-3') and PDE-42 [28]. Replacing the 5' end of PDEA4C with that of PDE4A7 generated the plasmid p19-Hyb1. The insert of p19-Hyb1 was purified as a *BamHI/SalI* fragment and cloned into pDNA3 for expression of the chimera, Hyb1.

To generate the Delta1 construct, the region encoding the C-terminal 86 amino acids of PDE4A4 was removed by PCR with primers pde4-pde1 and 3'Age (5'-TACCGGTCTCCCG-GGACGAGAA-3'). The PCR product was cloned into pCRscript and sequenced. A clone with no amino acid encoding changes was digested with *AgeI* and *HindIII* and ligated with the annealed oligonucleotides VSVa and VSVb. Following sequence analysis, the epitope-tagged C-terminal-deletion-encoding region was removed on an *XhoI/XbaI* fragment and ligated with *XhoI/XbaI*-digested pCMV6.1 to create pCMV-delta3vsv. To generate Delta4, the region encoding the C-terminal 14 amino acids unique to 2EL and hyb2-h6.1 was removed from pCMV-HYB2vsv using the primer pair pde4-pde1 and delta4-delta3' in a PCR. Delta4-Delta3' (5'-TGACCGGTTACCTGAGACTTCTCCACG-3') anneals with the sequence just upstream of where the C-terminal encoding regions of PDE4A7 and PDEA4C diverge. It contains a flanking *AgeI* restriction site that allows in-frame fusion with the VSV epitope tag used in the present study. The PCR product was cloned into pCRscript and sequenced to ensure that no sequence changes had occurred. One clone carrying no amino acid encoding changes was digested with *XhoI* and *AgeI*, and the insert was ligated with *XhoI/AgeI*-digested pCMV-HYB2vsv to replace the C-terminal encoding region of hyb2-h6.1 and create the plasmid pCMV-delta4vsv. To generate Delta3, the region encoding the C-terminal 86 amino acids found in all active PDE4A species was removed in a PCR with primers pde4-pde1 and 3'Age (5'-TACCGGTCTCCCGGGACGAGAA-3'). The PCR product was cloned into pCRscript and sequenced. A clone with no amino acid changes was digested with *AgeI* and *HindIII*, and ligated with annealed nucleotides VSVa and VSVb. Following sequence analysis, the epitope-tagged C-terminal-deletion-encoding region was removed on an *XhoI/XbaI* fragment and ligated with *XhoI/XbaI*-digested pCMV-6.1 to generate pCMV-HYB2vsv.

### DNA manipulation and sequencing

PCR was performed essentially as described previously [27,28,31,33], except that *Pfu* polymerase (Stratagene) was used instead of *Taq* polymerase. The reaction products were analysed by agarose gel electrophoresis and DNA fragments purified. Plasmid DNA was sequenced by the dideoxynucleotide chain termination method using T7 sequencing kits (Pharmacia) and [ $\alpha^{35}$ S]dATP (Amersham International). *Escherichia coli* strains K803 and NM522 were used for routine plasmid transformation and propagation. *E. coli* BB4 was used for library plating, whereas *E. coli* XL1-Blue and R408 helper phage were used for excision *in vivo*. The Bio-Rad gene pulser was used for transformation of yeast cells by electroporation. All PCR fragments cloned were subsequently sequenced to ensure that none of the plasmids used carried mutations arising from errors incorporated during PCR.

### Transfection of COS-7 cells and cell lysis for subfractionation and PDE assay

COS-7 cells were cultured, transfected and lysed as previously described by us in some detail in various other studies (for example, see [30,34–37]). Note that in subfractionation studies COS7 cells suspended in 3T3 lysis buffer (20 mM Hepes, pH 7.4, 50 mM NaCl, 50 mM NaF, 10% glycerol, 1% Triton X-100, 10 mM EGTA, 30 mM sodium pyrophosphate and protease inhibitors cocktail) were disrupted to allow for the preparation of P1 low-speed (1500 *g* for 10 min) pellet, P2 high-speed pellet and S2 high-speed supernatant fractions (100 000 *g* for 30 min), as described previously by us in some detail [30,34–37].

### SDS/PAGE and Western blotting

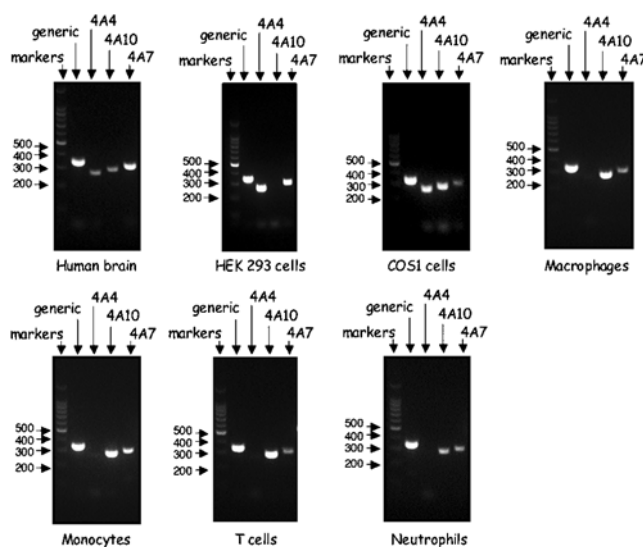
This was done as described previously [30,34–37]. The human PDE4A-specific polyclonal antiserum has been described in detail elsewhere by us [30,36], and was generated against a portion of the C-terminal region conserved in all active human PDE4A species and is represented by amino acids 788–886 of PDE4A4B. For detection of the VSV epitope (YTDIEMNRLGK), the monoclonal anti-P5D4 antibody was used as described previously by us for epitope-tagging PDE4 species [33]. Identification of the FLAG-epitope-tagged PDE4A7 construct was done using a specific monoclonal antibody (Sigma).

### PDE and protein assays

PDE activity was assayed by a modification of the two-step procedure of Thompson and Appleman [38], as described previously [39]. 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 for determination of activities with initial rates taken from linear time courses. Analysis of kinetic parameters was done as described in some detail previously [34–36], with dose-effect analyses done using 1  $\mu$ M cAMP as substrate. Protein was routinely measured by the method of Bradford [40], using BSA as a standard. Thermodenaturation analyses were done as described previously in some detail [26,41], using either a cytosolic (S2) high-speed supernatant soluble fraction or a total particulate fraction generated by centrifugation of disrupted cells at 100 000 *g* for 30 min, which is effectively a P1 + P2 fraction (see above).

### Laser-scanning confocal microscopy

COS cells were plated out on to coverslips (18 mm  $\times$  18 mm) at approx. 40% confluency 24 h prior to transfection with the required plasmid DNA. After transfection (48 h), cells were fixed for 10 min in 4% paraformaldehyde followed by three 5 min washes with 5 ml of PBS. Cells were then permeabilized by performing three 5 min incubations with 200  $\mu$ l of 0.2% Triton X-100 in Tris-buffered saline (TBS; 150 mM NaCl/20 mM Tris, pH 7.4), followed by a blocking step comprising three 5 min incubations with blocking buffer (20% appropriate serum and 4% BSA). The primary anti-VSV antibody was diluted to the required concentration in dilutant buffer (10% blocking buffer diluted with TBS) and 200  $\mu$ l of this mix was added to the slide and left to incubate at 20 °C for 2 h. Slides were then washed with three changes of blocking buffer and incubated with 200  $\mu$ l of 1:200 diluted Alexa-594-conjugated anti-rabbit IgG (Molecular Probes) for 1 h. Cells were then washed five times with TBS before mounting the coverslips on to microscope slides with Immunomount (Sandon). Cells were visualized using the Zeiss Axiovert 100 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany) using a Zeiss Plan-Apo 63  $\times$  1.4 NA



**Figure 2** Identification of transcripts for PDE4A7

RT-PCR was carried out on RNA obtained from the indicated cell lines/tissues, using primers specific for the indicated PDE4A isoform. Product sizes were PDE4A4 (257 bp), PDE4A10 (281 bp) and PDE4A7 (301 bp) using the primers described in the Experimental section. Generic indicates the results obtained using a primer pair designed to amplify a region common to all isoforms within that specific PDE4 subfamily. Such primer pairs are thus generic for a specific subfamily and able to detect whether there are transcripts for that specific PDE4 subfamily without any *a priori* knowledge of which particular isoforms are present. These results are typical of experiments carried out at least 3 times.

oil immersion objective. The Alexa-594-conjugated secondary antibodies were excited at 543 nm and detected at 590 nm.

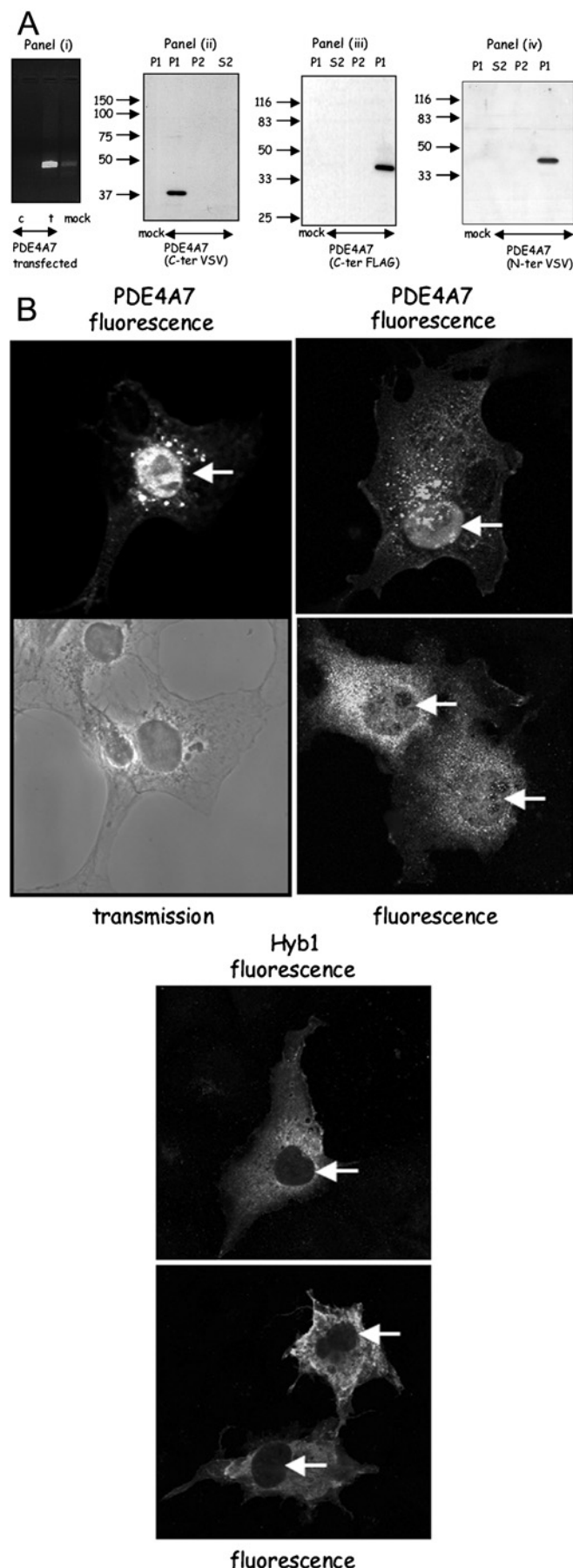
## RESULTS

### Transcripts for PDE4A7 are found in a diverse range of cell types

The *PDE4A* gene encodes the long PDE4A4B and PDE4A10 isoforms, the super-short PDE4A1 isoform [26,27] and the novel PDE4A7 isoform [28] (Figure 1). Although it is considered that PDE4A1 is expressed exclusively in brain, and PDE4A4B and PDE4A10 are widely expressed, nothing is known concerning the distribution of PDE4A7 [3,5]. In the present study we have isolated RNA from a variety of cell types and used RT-PCR, with isoform-specific primers, to show that transcripts for PDE4A7 are found in human brain and are also widely expressed in a range of human cell types, including macrophages, monocytes, T-cells and neutrophils, as well as in the model HEK293 and COS1 cells (Figure 2). Transcripts for PDE4A10 were found in all sources analysed, except HEK293 cells, whereas transcripts for PDE4A4 were not evident in blood-derived cell types (Figure 2). These results indicate cell-type-specific expression patterns for PDE4A isoforms.

### PDE4A7 lacks PDE activity

It has been suggested [28] that PDE4A7 may be catalytically inactive. This was surmised on the basis that it neither rescued the YMS5X *Saccharomyces cerevisiae* strain from heat-shock sensitivity, as did the active PDE4A4C, nor did it increase the PDE activity of YMS5X strain engineered to express PDE4A7. However, in these instances we were unable to ascertain expression of PDE4A7, as we had no antisera able to detect PDE4A7 [28]. In the present study, lysates of COS7 cells transfected with a



plasmid encoding PDE4A7, yielded a homogenate PDE activity of  $0.11 \pm 0.03$  nmol of cAMP hydrolysed/min per mg of protein (means  $\pm$  S.D.,  $n = 3$ ). This value was little different from that of either cells transfected with empty vector ( $0.12 \pm 0.04$  nmol of cAMP hydrolysed/min per mg of protein) or the non-transfected cells ( $0.13 \pm 0.03$  nmol of cAMP hydrolysed/min per mg of protein). In such PDE4A7-transfected cells we clearly identified a very greatly enhanced signal, compared with cells transfected with the empty vector, for transcripts detected by RT-PCR using PDE4A7-specific primers (Figure 3A, panel i). Ideally, we would have probed for PDE4A7 protein. However, despite numerous attempts, using both peptides and GST (glutathione S-transferase)-fusion proteins that reflect either all or part of the unique N- and C-terminal ends of PDE4A7, we have been singularly unsuccessful in trying to generate PDE4A7-specific antisera (results not shown). To obviate this problem we generated a PDE4A7 construct with a VSV-epitope tag at its C-terminus so that this chimera could be readily and specifically identified using an anti-VSV monoclonal antibody. This allowed the identification (Figure 3A, panel ii) of a single  $37 \pm 1$  kDa (means  $\pm$  S.D.,  $n = 5$ ) immunoreactive species in COS7 cells transfected to express the PDE4A7-VSV construct. However, the cAMP PDE activity of cells expressing this construct was, again, very similar ( $0.12 \pm 0.04$  nmol of cAMP hydrolysed/min per mg of protein; means  $\pm$  S.D.,  $n = 3$ ) to that of cells transfected with the empty vector. The addition of the PDE1 activator  $\text{Ca}^{2+}$ /calmodulin ( $50 \mu\text{M}$ ;  $20 \text{ ng/ml}$ ) did not increase cAMP PDE activity for PDE4A7-transfected cells above that seen in vector-only transfected cells ( $< 3\%$  difference), and assaying with  $1 \mu\text{M}$  cGMP as substrate showed no change in activity for PDE4A7-transfected cells above that seen in vector-only transfected cells ( $< 3\%$  difference). As a positive control, we transfected cells with a plasmid encoding PDE4A4C and recorded a cAMP PDE activity of  $6.2 \pm 0.2$  nmol of cAMP hydrolysed/min per mg of protein (means  $\pm$  S.D.,  $n = 3$ ; Table 1), which was some 50-fold greater than that seen in control cells. These analyses indicate that PDE4A7 is catalytically inactive as regards the hydrolysis of both cAMP and cGMP.

#### Analysis of cAMP PDE activity in chimera formed from N- and C-terminal domain swaps

The domain swaps found at both the N- and C-terminal regions of PDE4A7 encroach upon a region that is highly conserved amongst all PDE4 subfamilies (Figure 1A and [42]). This contains the catalytic unit, as implied from point mutations affecting catalysis [43] and inhibitor binding [44,45], as well as crystallographic analyses carried out on truncated PDE4 species with bound/docked competitive inhibitor [46,47].

**Figure 3** Expression of PDE4A7 in COS7 cells

(A) Panel (i): an RT-PCR analysis for PDE4A7 transcripts using isoform specific primers as in the Experimental section, with lanes showing control (c), test (t) and mock-transfected cells. RT-PCR was carried out to identify native levels of PDE4A7 transcripts. Panel (ii): the detection of C-terminal VSV-epitope-tagged PDE4A7 in subcellular fractions from transfected COS7 cells. Panel (iii): the detection of C-terminal FLAG-epitope-tagged PDE4A7 in subcellular fractions from transfected COS7 cells. Panel (iv): the detection of N-terminal VSV-epitope-tagged PDE4A7 in subcellular fractions from transfected COS7 cells. These results are typical of experiments carried out at least 3 times. (B) Typical laser-scanning confocal immunofluorescence analysis of COS cells transfected to express either VSV-tagged PDE4A7 (three examples) or VSV-tagged Hyb1 (two examples), as indicated. The picture labelled transmission relates to the fluorescence picture above it for PDE4A7. Identification of the localization of these species was done using an antibody specific for the VSV-epitope tag. No immunofluorescence whatsoever was detected for mock/empty-vector-transfected cells.

**Table 1** Activity analyses of PDE4A constructs

PDE activity (pmol of cAMP hydrolysed/min per mg of protein) and relative PDE activity was assessed in whole cell lysates for the indicated constructs expressed in COS7 cells. Relative PDE activity is the ratio of activity seen at  $V_{\max}$  conditions for equal immunoreactive amounts of enzymes.  $K_m$  values were determined on the cytosolic high-speed supernatant (S2) extracts from transfected cells using the range of substrate concentrations as given in the Experimental section. Results are expressed as the means  $\pm$  S.D. for  $n = 3$  separate experiments. n/a, not applicable, as enzyme shows no cAMP PDE activity.

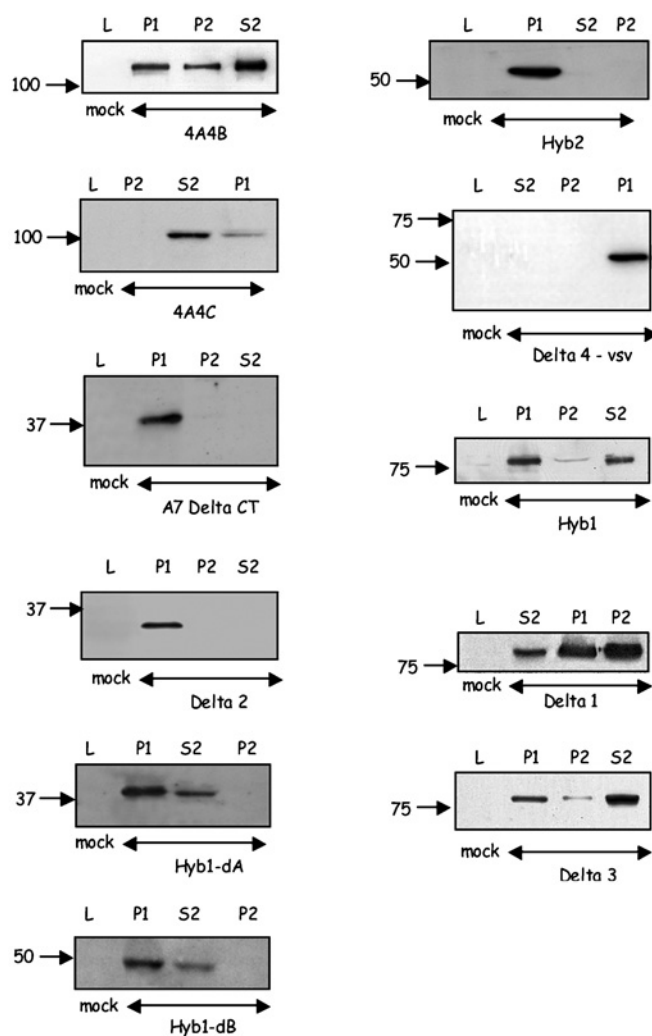
| Construct      | PDE activity    | Relative PDE activity | $K_m$ ( $\mu$ M) |
|----------------|-----------------|-----------------------|------------------|
| PDE4A4B        | $0.28 \pm 0.01$ | 1                     | $3.0 \pm 1.5$    |
| PDE4A4C (h6.1) | $6.2 \pm 0.2$   | $9.6 \pm 0.9$         | $4.0 \pm 1.0$    |
| PDE4A7 (2EL)   | Inactive*       | 0                     | n/a              |
| Hyb1           | $1.1 \pm 0.1$   | $1.7 \pm 0.2$         | $2.9 \pm 0.4$    |
| Hyb2           | Inactive*       | 0                     | n/a              |
| Delta1         | Inactive*       | 0                     | n/a              |
| Delta2         | Inactive*       | 0                     | n/a              |
| Delta4         | Inactive*       | 0                     | n/a              |

\* No detectable cAMP PDE activity, < 0.1 % the activity of PDE4A4.

Certain portions of PDE4 isoforms have been shown to exert inhibitory actions on the functioning of the catalytic unit [25,48]. However, removal of the unique C-terminal region of PDE4A7, as in the truncate A7-Delta-CT (Figure 1), failed to restore catalytic activity (Table 1). We also analysed the catalytically inactive Hyb2, formed by swapping the unique N-terminal region of PDE4A7 with that of the active PDE4A4C isoform through the construct Delta4, where the PDE4A7-unique N-terminal region was deleted (Figure 1). However, Delta4 showed no cAMP PDE activity (Table 1). Furthermore, Delta2, a truncate that lacked both the unique N- and C-terminal regions of PDE4A7 (Figure 1), was also catalytically inactive (Table 1). These results indicate that lack of catalytic activity in PDE47 is not due to any inhibitory effect of its unique regions.

We then set out to generate chimeras in order to evaluate whether one or other or both of the N- and C-terminal domain swaps in PDE4A7 account for its lack of catalytic activity (Figure 1). VSV-tagged Hyb2 (Figure 1) was generated to evaluate whether swapping the unique N-terminal region of PDE4A7 with that of the active PDE4A4C isoform could generate an active species. Hyb2 was identified as a single  $51 \pm 2$  kDa immunoreactive species (Figure 4) in transfected cells. However, when expressed at similar levels to PDE4A7-VSV, homogenate cAMP PDE activity ( $0.10 \pm 0.03$  nmol of cAMP hydrolysed/min per mg of protein;  $n = 3$ ) was, as with PDE4D7-VSV itself, similar to that seen for either non-transfected or empty vector-transfected cells (Table 1). Thus catalytic activity cannot be restored in PDE4A7 by replacing its N-terminal region with that of PDE4A4C.

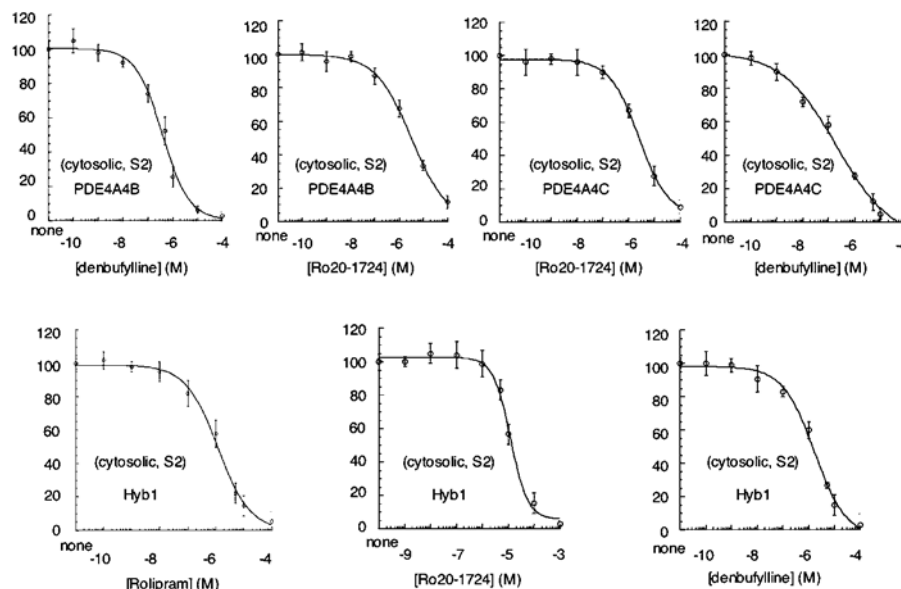
The chimera Hyb1 (Figure 1) was generated to see if swapping the unique C-terminal region of PDE4A7 with the C-terminal region found in all active PDE4A isoforms, such as PDE4A4C, could generate an active species. Hyb1 was identified, using anti-PDE4A serum, as a single  $82 \pm 3$  kDa immunoreactive species in transfected COS7 cells (Figure 4), which now showed an elevated PDE activity, compared with vector-only transfected cells, of  $1.1 \pm 0.1$  nmol of cAMP hydrolysed/min per mg of protein (means  $\pm$  S.D.,  $n = 3$ ). Thus replacing the unique C-terminal region of PDE4A7 with the C-terminal region found in all active PDE4A isoforms restored cAMP PDE activity (Table 1). Indeed, Hyb1 exhibited similar values for the  $K_m$  of hydrolysis by cAMP to both PDE4A4B and PDE4A4C (Table 1). However, whereas the relative  $V_{\max}$  value of PDE4A4C was some 10-fold higher than

**Figure 4** Expression of PDE4A chimeras and truncates in COS7 cells

COS7 cells were transfected with plasmids encoding the indicated constructs. Cells were then disrupted and subjected to differential centrifugation to generate the P1 and P2 pellet fractions, as well as the S2 high-speed supernatant fraction. In addition to this, lysates from empty-vector-transfected cells are shown. Each lane was run with 50  $\mu$ g of protein. Identification was done using an antiserum specific for the common C-terminal region found in all active PDE4A isoforms to detect PDE4A4B (4A4B) and PDE4A4C (4A4C). All other species were detected using a VSV antibody to detect the epitope tag. The results are typical of experiments carried out at least 3 times with different transfections.

that of PDE4A4B, that of Hyb1 was only about 2-fold higher (Table 1). Thus lack of catalytic activity in PDE4A7 is due to the absence of an appropriate C-terminal portion attached to the common PDE4A 'core' (solid black boxes in constructs shown in Figure 1).

In characterizing Hyb1 further, we demonstrated that the soluble, cytosolic form was dose dependently inhibited by the archetypal PDE4 selective inhibitor rolipram (Figure 5), with a similar  $IC_{50}$  value to those values reported previously [36] for both PDE4A4B and PDE4A4C, and as we also determined in the present study (Table 2). Cytosolic Hyb1, PDE4A4B and PDE4A4C were also all dose dependently inhibited (Figure 5 and Table 2) by the PDE4 selective inhibitors, Ro-20-1724 and denbufylline [12,14,49]. However, compared with both PDE4A4B and PDE4A4C, Hyb1 exhibited a somewhat higher  $IC_{50}$  value for inhibition, in particular as regards to Ro-20-1724



**Figure 5** Inhibition of PDE4 species by selective inhibitors

COS7 cells were transfected with plasmids encoding the indicated constructs. These were the long PDE4A4B isoform, the truncated PDE4A4C species and the active chimera, Hyb1. PDE activity in the cytosolic, S2 fraction was determined with 1  $\mu$ M cAMP as substrate and the percentage change shown occurring upon challenge with the indicated concentrations of the stated PDE4-selective inhibitors. Results are shown for experiments carried out three times with the different transfections (means  $\pm$  S.D.,  $n = 3$ ).

**Table 2** Sensitivity of Hyb1 to PDE4 selective inhibitors

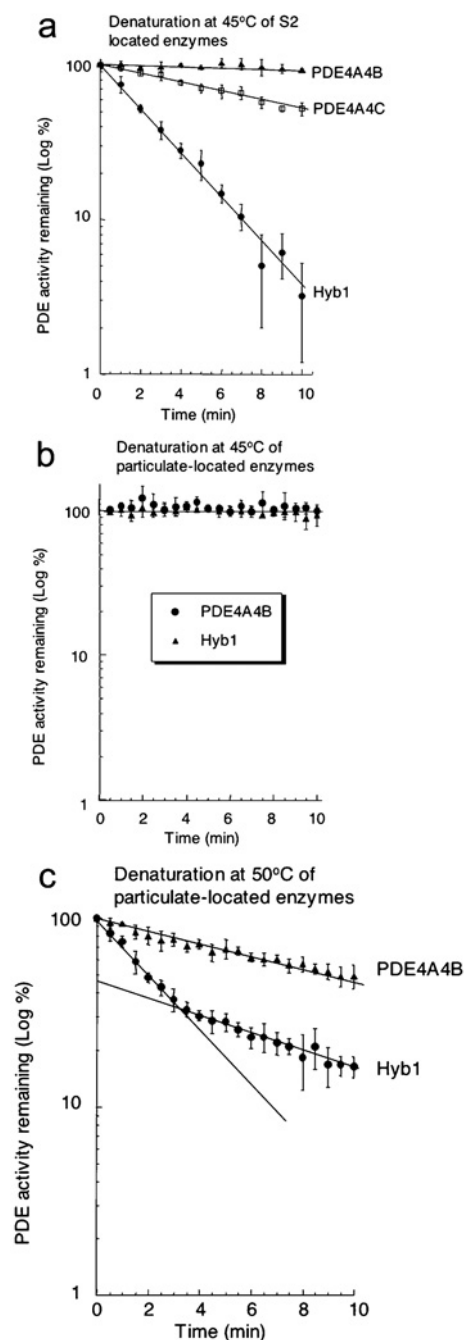
The indicated constructs were expressed in COS7 cells and then both the high-speed supernatant (S2) and high-speed pellet (P2) fractions taken for analysis. PDE activity was determined using 1  $\mu$ M cAMP as substrate using the range of inhibitor concentrations indicated in the Experimental section and in Figure 5. The PDE activity of these fractions is given in Table 1. Results are expressed as the means  $\pm$  S.D. for  $n = 3$  separate experiments. n/a, not applicable, as PDE4A4C is not found in the P2 fraction.

| Construct | Inhibitor ...<br>Fraction ... | IC <sub>50</sub> ( $\mu$ M) |               |                |               |                 |                |
|-----------|-------------------------------|-----------------------------|---------------|----------------|---------------|-----------------|----------------|
|           |                               | Rolipram                    |               | Ro-20-1724     |               | Denbutylline    |                |
|           |                               | S2                          | P2            | S2             | P2            | S2              | P2             |
| PDE4A4B   |                               | 1.1 $\pm$ 0.2               | 0.3 $\pm$ 0.1 | 2.8 $\pm$ 0.2  | 1.9 $\pm$ 0.8 | 0.39 $\pm$ 0.05 | 0.37 $\pm$ 0.1 |
| PDE4A4C   |                               | 0.78 $\pm$ 0.08             | n/a           | 2.3 $\pm$ 0.3  | n/a           | 0.21 $\pm$ 0.03 | n/a            |
| Hyb1      |                               | 2.2 $\pm$ 0.3               | 0.9 $\pm$ 0.1 | 11.2 $\pm$ 1.5 | 3.5 $\pm$ 0.9 | 1.01 $\pm$ 0.06 | 0.23 $\pm$ 0.1 |

(Table 1). We also evaluated the ability of the P2 pellet-associated Hyb1 to be inhibited by these various compounds (Table 2). Interestingly, in each instance, the P2 pellet-associated form of Hyb1 appeared to be slightly more sensitive to the action of these compounds compared with the cytosolic form (Table 2). Indeed, the IC<sub>50</sub> values for inhibition of P2 pellet-associated form of Hyb1 by Ro-20-1724 and denbutylline were more akin now to those exhibited by the full-length PDE4A4B (Table 2).

Differences in sensitivity to thermal denaturation have been employed to detect altered stability of proteins and thus to infer alterations in conformation. As denaturation occurs as a first-order process, the resulting exponential decay in activity is most simply analysed as a semi-log plot of the percentage loss in activity with time, from which a half-life ( $t_{1/2}$ ) can readily be determined. For example, this has been used to demonstrate that the unique N-terminal regions of the long PDE4A4C and PDE4A10 isoforms exert different actions on the conformational status of their identical catalytic units [26]. In the present study we see that the activity of cytosolic (S2 fraction) Hyb1 decays as a single exponential at 45 °C (Figure 6a), with a  $t_{1/2}$  of 2.1  $\pm$  0.7 min (mean  $\pm$  S.D.,  $n = 3$  separate experiments). In contrast to this, the

activity of cytosolic (S2 fraction) PDE4A4C is considerably more thermostable (Figure 6a) and decays with a  $t_{1/2}$  of 10.1  $\pm$  1.7 min (mean  $\pm$  S.D.,  $n = 3$  separate experiments), whereas cytosolic (S2 fraction) PDE4A4B is even more thermostable with little change (< 8%;  $t_{1/2}$  > 80 min) evident in its activity occurring over 10 min at 45 °C (Figure 6a). Such data suggest that the distinct N-terminal portions of these enzymes exert selective influences on the conformation, and thus functioning, of the catalytic unit. Consistent with this, we made the striking observation that N-terminal truncation of Hyb1, so as to delete the region unique to PDE4A7, led to loss of catalytic activity, as seen in the Delta1 construct (Figure 1 and Table 1). The N-terminal portions of PDE4A isoforms are critically involved in membrane targeting [23,25,26,30,36,37]. Interestingly, we see here that in marked contrast with the soluble form of Hyb1 (Figure 6a), the particulate-associated form of Hyb1 is stable to heat at 45 °C over a period of 10 min, as indeed is the soluble form of the full-length isoform, PDE4A4B (Figure 6b). This indicates that particulate association of Hyb1 engenders a conformational change in the enzyme resulting in increased thermostability. In order to determine if there were differences in thermostability



**Figure 6** Thermal denaturation of PDE4A species

The thermodenaturation of the activity of the indicated PDE4A species given as a log % of the starting activity (100%) is shown. Thermal inactivation of a single entity shows exponential decay, yielding linear semi-log plots of the Log<sub>10</sub> percentage of activity remaining versus time. (a) Rhe cytosolic (S2 fraction) forms of Hyb1, PDE4A4B and PDE4A4C were analysed after treatment for the indicated periods at 45 °C prior to being taken for assay of cAMP PDE activity with 1  $\mu$ M cAMP as substrate. (b and c) The total particulate (P1 + P2 fraction) of Hyb1 and PDE4A4B are analysed subsequent to exposure for the indicated times at either (b) 45 °C or (c) 50 °C. The results are expressed as the means  $\pm$  S.D. for 3 individual experiments at each time point.

between the particulate-associated forms of Hyb1 and PDE4A4B, we analysed their thermostability at a higher temperature, namely 50 °C (Figure 6c). Although this clearly identified the particulate-associated form of Hyb1 as being more thermolabile than that of PDE4A4B, it is evident that the activity-decay plot of the

particulate-associated form of Hyb1 is clearly biphasic (Figure 6c). This is most simply interpretable as two components, a more thermolabile species with a  $t_{1/2}$  of  $2.2 \pm 0.4$  min, which provides around 50% of the total activity, and a more thermostable species with a  $t_{1/2}$  of  $6.9 \pm 0.5$  min (mean  $\pm$  S.D.,  $n = 3$  separate experiments), which provides the other half of the total activity. As particulate association clearly affects the thermostability of Hyb1, such a biphasic plot is indicative of two Hyb1 subpopulations that perhaps reflect different modes of particulate-association, with distinct consequences for the conformation of anchored Hyb1. Interestingly, the  $t_{1/2}$  of the more thermostable species of Hyb1 had similarity with that of the particulate-associated form of PDE4A4B, whose  $t_{1/2}$  was  $9.1 \pm 0.4$  min (mean  $\pm$  S.D.,  $n = 3$  separate experiments). These results clearly show that particulate association has consequences for the conformation of PDE4A isoforms.

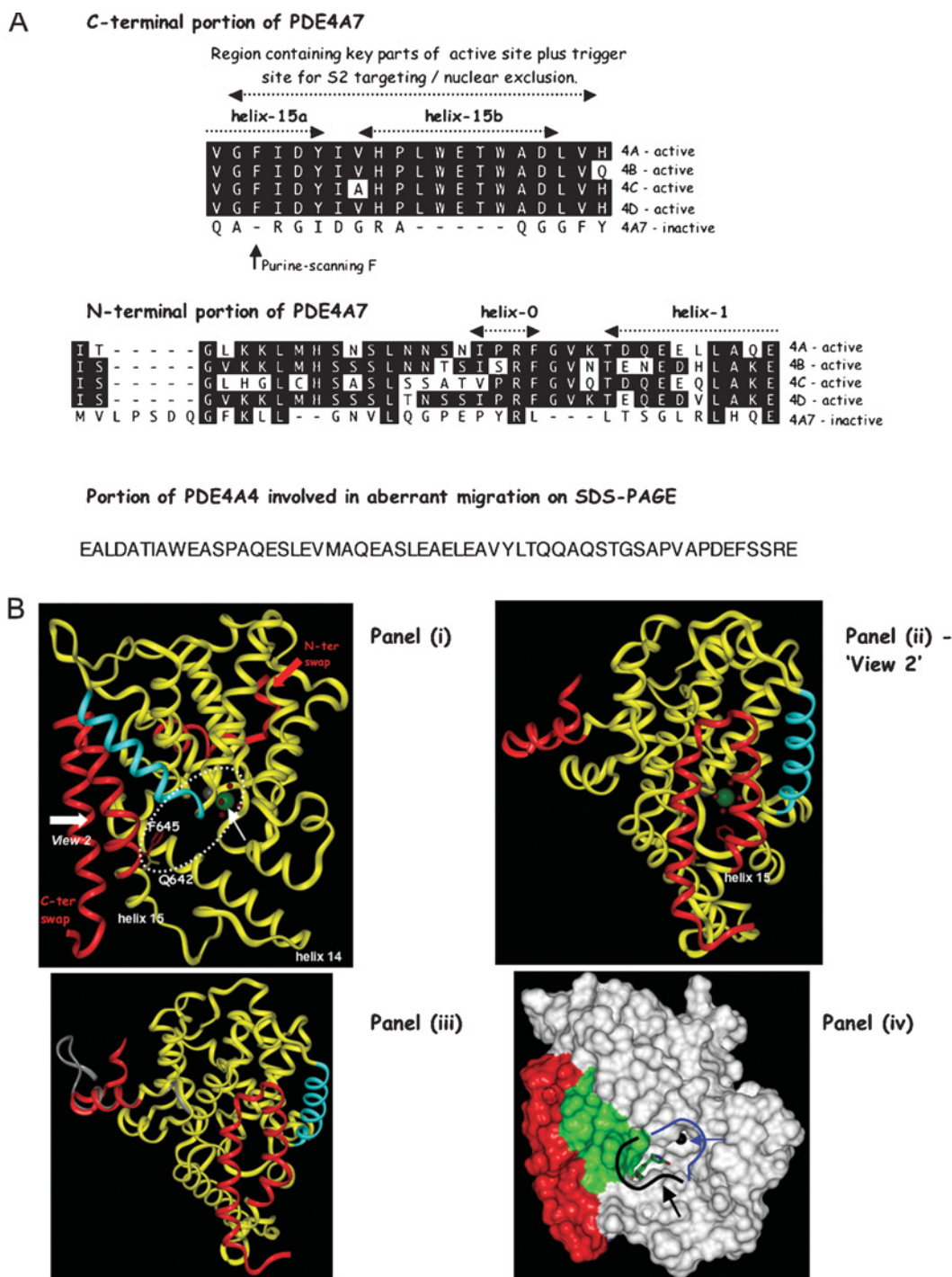
Thus it would seem that the common PDE4A 'core' (Figure 1, region shown shaded in black) requires not only appropriate C-terminal sequence, but also appropriate N-terminal sequence (Figures 7A and 7B) in order to be catalytically active. It is, then, fascinating to find here that such functionality can be provided not only by N-terminal sequence found in active PDE4A4 isoforms from all four subfamilies, but also by the unique N-terminal region of PDE4A7, as seen in Hyb1 (Table 1, Figures 1 and 7).

#### Migration of PDE4A species on SDS/PAGE

Active human and rat PDE4A isoforms migrate anomalously on SDS/PAGE, resulting in apparent molecular masses that are some 10–20 kDa larger than those predicted from primary amino acid sequences [5,20,26,27,36,37]. In profound contrast to this, the VSV- and FLAG-tagged forms of PDE4A7 migrated on SDS/PAGE as predicted based upon their primary sequences (Table 3 and Figure 4). Similarly, the PDE4A7 truncates, A7-Delta-CT and Delta2, also migrated on SDS/PAGE as predicted from their primary sequence (Table 3 and Figure 4). These results suggest that N/C-terminal regions of active PDE4A species underpin their anomalous migration. However, Hyb2, a chimera formed by swapping the unique N-terminal region of PDE4A7 with that of the active PDE4A4C form, migrated on SDS/PAGE at the size predicted from its primary sequence (Table 3 and Figure 4). In marked contrast, however, the chimera Hyb1, formed by swapping the unique C-terminal region of PDE4A7 with that found in active PDE4A isoforms, migrated aberrantly on SDS/PAGE at around 11 kDa higher than that predicted (Table 3 and Figure 4). These results indicate that the anomalous migration of active PDE4A isoforms on SDS/PAGE is due to an effect exerted by their common C-terminal region. In order to obtain a better approximation of the portion of the C-terminal region that leads to aberrant migration on SDS/PAGE, we generated the C-terminal truncates Hyb1-dA, which deletes 224 residues, and Hyb1-dB, which deletes 142 residues (Figure 1), only to find that both of these migrated as predicted by their primary amino acid sequence on SDS/PAGE (Table 3). In contrast, the Delta3 truncate, where 86 amino acids were deleted from the C-terminus, migrated aberrantly on SDS/PAGE (Table 3 and Figure 4). Thus the region located between Glu<sup>744</sup> and Glu<sup>800</sup>, numbered here as in the full-length active PDE4A4B isoform, is involved in determining the aberrant migration of active PDE4A isoforms on SDS/PAGE (Figures 1 and 7).

#### Intracellular distribution of PDE4A7

Expressed in transfected COS cells, the PDE4A4B and PDE4A4C forms are predominantly localized to the high-speed supernatant (S2) fraction [30,36], as we confirm in the present study (Table 3).



**Figure 7** Unique N- and C-terminal regions of PDE4A7

(A) The sequences of the unique N- and C-terminal portions of PDE4A7 together with the 'equivalent' regions of active PDE4 forms from all four PDE4 subfamilies are shown. (B) The structure of the conserved catalytic region of PDE4B (panels i and ii) are shown, where the regions highlighted in red are equivalent to those replaced by the N- and C-terminal domain swaps occurring in PDE4A7 and the region highlighted in blue is helix-12. The dotted white ring outlines the region of the catalytic site with the bound  $Mg^{2+}$  (green sphere) indicated by a white arrow. Panel (iii): a comparison of the structures of the core catalytic unit of PDE4B compared with that of PDE4D. Where the extreme N-terminal portion differs with that of PDE4D this is shown in grey, for PDE4D. PDE4B is shown in yellow/red. Panel (iv): a surface rendition of the catalytic unit of the determined crystal structure of PDE4D with rolipram in the active site, the distal portion of which is marked by the black curve as indicated by the black arrow. The proximal limit of the catalytic site is delineated in blue with bound  $Mg^{2+}$  (black sphere) indicated by a blue arrow. The region shown in red is the region equivalent to that deleted in PDE4A7. The region shown in green is the equivalent of that altered with novel C-terminal sequence in PDE4A7 and clearly shows how it affects the distal portion of the catalytic site.

Studies from various laboratories have shown [3,5,20,21] that N-terminal truncation to remove the isoform-specific portion, as well as UCR1 and UCR2, generates a soluble species found en-

tirely in the S2 high-speed supernatant fraction. It thus came as a complete surprise to discover that the C-terminal VSV-tagged PDE4A7 was localized entirely to the P1 pellet fraction when

**Table 3 Molecular mass of PDE4A constructs and their subcellular distribution in COS7 cells**

Sizes were predicted on the basis of their primary sequence. The observed size was derived from SDS/PAGE analyses carried out under denaturing conditions. Results are expressed as the means  $\pm$  S.D. for  $n = 3$  separate experiments.

| Construct      | Molecular mass (kDa) |             | Subcellular distribution (%) |            |            |
|----------------|----------------------|-------------|------------------------------|------------|------------|
|                | Predicted            | Observed    | P1                           | P2         | S2         |
| PDE4A4B        | 99                   | 125 $\pm$ 4 | 23 $\pm$ 6                   | 17 $\pm$ 6 | 60 $\pm$ 4 |
| PDE4A4C (h6.1) | 76.4                 | 102 $\pm$ 3 | 20 $\pm$ 4                   | < 2        | 80 $\pm$ 4 |
| PDE4A7 (2EL)*  | 38.4                 | 37 $\pm$ 2  | 100                          | < 2        | < 2        |
| Hyb1           | 61.2                 | 82 $\pm$ 3  | 64 $\pm$ 5                   | 6 $\pm$ 4  | 30 $\pm$ 6 |
| Hyb2*          | 53.6                 | 52 $\pm$ 2  | 100                          | < 2        | < 2        |
| Delta1         | 57.6                 | 80 $\pm$ 3  | 42 $\pm$ 3                   | 38 $\pm$ 3 | 20 $\pm$ 4 |
| Delta2*        | 33.4                 | 31 $\pm$ 3  | 100                          | < 2        | < 2        |
| A7-Delta-CT*   | 36.8                 | 36 $\pm$ 3  | 100                          | < 2        | < 2        |
| Hyb1-dA*       | 39.1                 | 39 $\pm$ 4  | 71 $\pm$ 9                   | < 2        | 29 $\pm$ 9 |
| Hyb1-dB*       | 48.4                 | 48 $\pm$ 4  | 73 $\pm$ 4                   | < 2        | 27 $\pm$ 4 |
| Delta3*        | 69.6                 | 81 $\pm$ 2  | 17 $\pm$ 3                   | 5 $\pm$ 3  | 78 $\pm$ 3 |
| Delta4*        | 52.1                 | 52 $\pm$ 3  | 100                          | < 2        | < 2        |

\* These constructs were C-terminally tagged with the VSV epitope of 14 amino acids and thus the calculated predicted sizes given here are 1.6 kDa larger than those of the untagged species.

expressed in COS cells (Figure 3A, panel ii, and Table 3). We were concerned that the type of epitope tag used might have affected such localization, so we generated a C-terminal FLAG-tagged PDE4A7 and showed that it too was found only in the P1 fraction (Figure 3A, panel iii). In case distribution was affected by the C-terminal placing of the tag, we also generated a N-terminal VSV-tagged PDE4A7. This, again, was localized entirely to the P1 pellet fraction in COS cells (Figure 3A, panel iv). Consistent with the lack of PDE4A7 in the cytosol, analysis of the localization of VSV-tagged PDE4A7 in COS cells by confocal immunohistochemistry showed it to be associated with particulate structures in these cells (Figure 3B). In many instances these were concentrated around the nucleus. In contrast to this, Hyb1 immunofluorescence was completely different, being more akin to that observed previously for the active full-length isoform PDE4A4 [30] and its rodent homologue PDE4A5 [37], being spread throughout the cell cytosol and with some structured association consistent with a fraction being pellet-associated (Figure 3B). Additionally, although we noted that both Hyb1 (Figure 3B) and PDE4A4 [30] were, seemingly, excluded from the nucleus, a fraction of PDE4A7 was located within the nucleus (Figure 3B).

We then went on to determine that A7-Delta-CT, which lacks the unique C-terminal portion of PDE4A7 (Figure 1), was exclusively associated with the P1 fraction (Figure 4 and Table 3). Furthermore, the Delta2 construct, which lacks both of the unique N- and C-terminal portions of PDE4A7, was exclusively associated with the P1 fraction (Figure 4 and Table 3). Thus targeting to the P1 fraction appears not to be associated with the unique portions of PDE4A7, but is a property associated with the 'core' PDE4A region (Figure 1, solid black boxes).

These data suggest that there must be a region in active PDE4A isoforms that prevents the exclusive P1 targeting and allows at least a fraction of these isoforms to be found in the S2 fraction, as noted for the PDE4A4B and PDE4A4C species (Table 3, Figure 4 and [30,36]). Given that a major fraction of the active PDE4A4C isoform is localized to the S2 fraction (Table 3, Figure 4 and [30,36]), we analysed the Hyb2 chimera, where the N-terminal portion of PDE4A4C was used to replace that of PDE4A7 (Figure 1). Surprisingly, we found that Hyb2 was also

exclusively associated with the P1 pellet fraction (Figure 4 and Table 3). This suggests that the C-terminal portion found common to all active PDE4A isoforms contains a signal allowing a portion of these enzymes to accrue in the S2 fraction. To address this, we analysed the distribution of the chimera Hyb1, which has the N-terminal portion of PDE4A7 and the entire C-terminal region found in common to all active PDE4A isoforms (Figure 1). In doing this we found that Hyb1 accrued in both the S2 and P1 fractions, rather than exclusively in the P1 fraction (Figure 4 and Table 3), indicating a role for the C-terminal portion found common to all active PDE4A isoforms. Analysis of the intracellular distribution of various C-terminal truncates (Figures 1 and 4, Table 3) allowed this to be further refined. Thus the Delta1 construct, where the 'core' region common to both PDE4A7 and active PDE4A isoforms is fused to a truncated C-terminal region (Figure 1), is clearly distributed in the S2 and P2 fractions, as well as in the P1 fraction (Figure 4 and Table 2). Such analysis of Delta1 not only shows that targeting outside the P1 fraction is achieved by the C-terminal portion of catalytically active PDE4A isoforms, but that the C-terminal 86 amino acids of PDE4A4 are not needed to achieve this as they have been truncated from Delta1 (Figure 1). None of the Hyb1-dA, Hyb1-dB and Delta3 truncates were exclusively localized to the P1 fraction, but the Delta4 truncate was (Figure 4 and Table 3). This indicates that a 20-amino-acid region (643–662 of PDE4A4) (Figure 1) located in the C-terminal region of all active PDE4A isoforms is required to prevent their exclusive targeting to the P1 particulate fraction.

## DISCUSSION

To date, PDE4A7 is the only PDE4 isoform to arise from both 5' and 3' domain swaps, giving it both a unique 32-residue N-terminal region and a unique 14-residue C-terminal region [27,28]. PDE4A7 was originally cloned from a Jurkat T-cell cDNA library, with subsequent analysis of the human *PDE4A* gene locus identifying consensus splice sites for PDE4A7 generation, indicating that PDE4A7 is an authentic gene product [27,28]. In the present study we identify transcripts for PDE4A7 in human brain and in a range of human cell types, including macrophages, monocytes, T-cells and neutrophils, as well as in the model HEK293 and COS1 cells (Figure 2). Amongst the spectrum of blood-derived cell types studied here (Figure 2), it seems that the expression of PDE4A7 may vary relative to that of the long PDE4A4B and PDE4A10 isoforms. This would be consistent with the notion [3,50] that distinct promoters control the expression of individual isoforms, as has been shown for short PDE4D isoforms [3,50] and the long PDE4A10 [26] and PDE4D5 isoforms [51].

We show here that recombinant PDE4A7, expressed in COS7 cells, is clearly unable to hydrolyse cAMP (Table 2), making it unique amongst PDE isoforms. Interestingly, however, there is a growing appreciation that certain genes can express catalytically inactive isoforms [52,53]. This notion has gained credence recently from studies done on the protein kinase superfamily [52,53]. For example, the catalytically inactive ErbB3 is a member of the Erb family of protein tyrosine kinases [54] and there are indications that it may serve as a signalling scaffolding protein [55]. Similarly, KSR (kinase Suppressor of Ras) is a catalytically inactive 'protein kinase' that plays a scaffolding role in the mitogen-activated-protein-kinase signalling processes [56]. Indeed, it is thought that the human genome may encode as many as 50 forms of catalytically inactive 'protein kinases' [52]. Nevertheless, the full extent and significance of such phenomena has yet to be realized and determining the functional role of such

species poses a formidable technical challenge [29]. In addition, alternative splicing of mRNA is often used as a regulatory switch, determining whether a functional protein is made or not. Interestingly, in this regard, catalytically inactive splice variants of plasma cholesteryl ester transfer protein have been recognized that complex with the active form and prevent its secretion by cells [57]. Furthermore, although the Wilms' tumour suppressor protein WT1 has been shown to self-associate, these oligomers form much less efficiently than those formed between mutant truncated forms of WT1 and the full-length protein [58]. Given that PDE4 isoforms appear to be capable of forming dimers and oligomers under certain conditions [20,46,59,60], this might point to a role for PDE4A7 in interacting with other PDE4 isoforms and affecting their intracellular targeting and functioning.

As a start to appreciate the role of PDE4A7, we set out in the present study to determine the basis of its clear lack of catalytic activity. In this regard, it has been noted that N-terminal portions of various active PDE4 isoforms can exert an inhibitory effect on catalytic activity [20,21,25,37,43], suggesting the possibility that the N- and C-terminal regions unique to PDE4A7 might cause an overwhelming inhibitory effect on its catalytic function. However, deletion of such regions singularly failed to generate active enzymes (Table 1). In marked contrast, the chimera Hyb1, generated to replace the unique C-terminal portion of PDE4A7 with that found in active PDE4A species, was clearly active, whereas the chimera Hyb2, providing a comparable N-terminal domain swap, was not (Table 1). Thus lack of catalytic activity in PDE4A7 is clearly due to the absence of key residues/structures from its C-terminal region.

Comparison of the sequence of active isoforms from each of the four PDE4 subfamilies has identified a highly similar region that is shown in Figure 1 as the 'PDE4 conserved catalytic region' [5,20]. This region of PDE4B and PDE4D has been crystallized and shown to form a compact unit comprising three subdomains [46,47]. The catalytic site within this unit was identified from the binding of competitive inhibitors [46,47], together with studies done on inactivating point mutants [43,61,62], and inferences made concerning a proposed mechanism based upon the disposition of groups within the putative active site [20]. It can be seen (Figures 7A and 7B) that the C-terminal domain swap in PDE4A7 causes loss of part of helix-15a found in active PDE4 enzymes and which contains a phenylalanine residue [Phe<sup>645</sup> in PDE4A4B; Figures 7A and 7B, panel i] considered crucial in providing aromatic stacking and hydrophobic interactions with the cAMP purine ring in the substrate complex [20,46,47]. Thus loss of this residue (Figure 7B, panel i) and disruption of the distal portion (Figures 7A and 7B, panel iv) of the catalytic site itself in such a domain swap would certainly be consistent with lack of catalytic activity in PDE4A7. Figure 7(B, panel iv) shows the active site of PDE4D, with rolipram located within the distal portion. The region shown in red is the equivalent of that deleted in PDE4A7, whilst that in green is the equivalent of that altered in the 3' domain swap in PDE4A7. It is interesting to speculate that, assuming no gross deformation occurs in the folding of the rest of PDE4A7, a binding site may still remain, albeit not for cyclic nucleotides. Indeed it may even show PDE activity with novel substrate specificity. Further work, including structural analyses, will be needed to evaluate this.

Analyses of the chimera Hyb1 and the truncate Delta1 (Figure 1) allow us to show that the N-terminal portion of the core catalytic domain, which provides helix-0 and part of helix-1 [46,47] in the first subdomain of the catalytic unit (Figures 7A and 7B, panels i–iii), is also essential for catalytic function. This region is not well conserved amongst various PDE families and clearly lies outside of the so-called PDEase domain, identified

by domain search engines as conserved in the PDE superfamily [63]. However, this N-terminal portion, which provides the helix-0–helix-1 region, is clearly highly conserved within all four PDE4 subfamilies [5] (Figure 7A). Inspection of the PDE4 crystal structure [46,47] does not immediately suggest a role for the helix-0–helix-1 region, which lies on the opposite side of the catalytic unit to the active site (Figure 7B, panels i–iii). However, by packing on to the periphery of the core catalytic domain it may serve to stabilize a catalytically functional tertiary structure. Indeed, although comparative analysis of the PDE4B and PDE4D catalytic unit structures [46,47] identified some difference in the folding of sequence preceding helix-1, the folding of helix-1 itself is conserved between the two structures (Figure 7B, panel iii). Surprisingly, however, we show here that the unique N-terminal region of PDE4A7 is able to support catalytic activity, as exemplified in the Hyb1 chimera (Figure 1 and Table 1). Comparison of the sequence of this region of PDE4A7 with that equivalently located in active PDE4 isoforms does not show high conservation, although we do note a conserved LxxE motif (Figure 7A). However, while it is clear that the unique N-terminal region of PDE4A7 does provide sufficient structural information to allow for the formation of an active catalytic site, it clearly influences the conformation of the catalytic unit in a rather different way from that of the N-terminal region of the PDE4A4B long isoform. This is evident from striking differences seen in the thermostability of the activity of these enzymes (Figure 6), and also in differences in their susceptibility to inhibition by Ro-20-1724 and denbufylline (Table 2). These results suggest that comparative structural studies of the catalytic unit coupled to the unique N-terminal region of PDE4A7 compared with the equivalent N-terminal portion of the catalytic unit found in active PDE4 isoforms (Figure 7A) may provide novel insights into the role of the N-terminal portion of subdomain 1 of the catalytic unit in stabilizing an active entity. The importance of N-terminal portion of subdomain 1 to catalytic function suggests that it could provide a surface for regulating the functioning of the catalytic site. This may be of significance in that it is linked to the regulatory module provided by UCR1 and UCR2, which serves to orchestrate the functional response of the catalytic unit when PDE4 isoforms are phosphorylated by PKA (protein kinase A) [64,65] and by ERK (extracellular-signal-regulated kinase) [66–68].

Whereas active PDE4A isoforms migrate anomalously on SDS/PAGE with apparent molecular masses some 10–20 kDa greater than predicted from their primary amino acid sequence [5,42], this is not the case for PDE4A7, which migrates as predicted (Table 2). However, analyses of the Hyb1 and Hyb2 chimeras identify the C-terminal portion of active PDE4A isoforms as determining their anomalous migration on SDS/PAGE (Table 2 and Figure 4). This was further refined, using C-terminal truncation (Figures 1 and 4, Table 2), so as to identify a 56-amino-acid region, located between Glu<sup>744</sup> and Glu<sup>800</sup> in PDE4A4B (Figures 1 and 7A), as containing residues that underpin this effect. The precise basis of this effect remains to be identified. However, these results point to the possibility that the C-terminal portion of active PDE4A isoforms might exert conformational effects and consequent functional actions on active PDE4 isoforms.

When expressed in COS cells, PDE4A4B and PDE4A4C are predominantly found in the S2 high-speed supernatant, cytosolic fraction [30,36] (Table 2). It came as a considerable surprise to find that PDE4A7 was exclusively localized to the P1 pellet fraction (Figure 3B and Table 3). Such targeting was clearly not due to the novel N- and C-terminal portions of PDE4A7, as truncates that lacked either both or just one of these portions showed exclusive P1 targeting (Figure 3 and Table 3). Intriguingly, however, whereas the chimera Hyb2 was exclusively localized to

the P1 pellet fraction, the chimera Hyb1 was found in both the cytosol and particulate fractions (Table 3 and Figure 4). This indicates that the C-terminal region of active PDE4A species prevents the exclusive P1 targeting seen with PDE4A7. Analysis of C-terminal truncates (Table 3 and Figure 4) identifies the same C-terminal 19-amino-acid region that provides part of the active site in active PDE4A isoforms (643–662 of PDE4A4) (Figure 1) as containing information to prevent the exclusive targeting of these active PDE4 species to the P1 particulate fraction. This helix-15a–helix-15b region, found in active PDE4 isoforms, is particularly enriched in aromatic and hydrophobic amino acids (Figure 7A), which underpins their self-interaction and association with helix-12 (Figure 7B) [46,47]. Thus the short C-terminal replacement sequence in PDE4A7 can be expected to have profound consequences on the tertiary structure of the catalytic unit, extending beyond simply causing loss of catalytic function by removing Phe<sup>645</sup>. Indeed, because of this, we may expect to see altered folding of helix-12, as well as the residual helix-15a and the long connecting loop to helix-14 in PDE4A7 (Figure 7B). The exclusive targeting of PDE4A7 to the P1 fraction may reflect one consequence of such altered conformation. Clearly it generates a unique pattern of intracellular distribution on transfected COS cells, being associated primarily with punctate intracellular structures and, seemingly, also able to enter the nucleus (Figure 3B). Given that a key property of a variety of PDE4 isoforms is to interact with other proteins in order to exert specific intracellular actions (for example, see [23,62,69,70]), it seems likely that the targeting of PDE4A7 to the P1 fraction is indicative of an interaction with an anchor protein. However, the difficulties in identifying novel partners for particulate-associated proteins are formidable, not least for those for which antiserum is not available.

These studies provide novel insights into the complex molecular machinery that comprises the PDE4 catalytic unit. In particular they show that the domain swap caused by the novel 3' splicing in PDE4A7 results not only in loss of catalytic function, but also in a conformational change of the core region of PDE4A7, of which one functional outcome is to confer the exclusive targeting of PDE4A7 to the P1 particulate fraction. That aberrant migration of active PDE4A isoforms on SDS/PAGE is due to a portion of their C-terminal region may indicate that this, as well as N-terminal portions of PDE4 isoforms, also has a functional role. Finally, we show that the helix-0–helix-1 region is required for generating a catalytically functional enzyme and that the unique N-terminal domain of PDE4A7 can substitute for the helix-0–helix-1 region conserved in active isoforms from all four PDE4 subfamilies. The delineation of these properties of the unique PDE4A7 isoform provide a foundation for the challenging studies that will be needed to appreciate the functional role of this widely expressed PDE4A isoform.

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