Biochemical and Biophysical Research Communications **272,** 186–192 (2000)
doi:10.1006/bbrc.2000.2743, available online at http://www.idealibrary.com on IDEAL®

Cloning and Characterization of the Human and Mouse PDE7B, a Novel cAMP-Specific Cyclic Nucleotide Phosphodiesterase

Clare Gardner,* Nicola Robas,* Darren Cawkill,† and Mark Fidock*,[1]
**Department of Genetic Technologies and †Hit Discovery Group, Pfizer Central Research, Sandwich, Kent, CT13 N9J, United Kingdom*

Received April 18, 2000

We have identified and characterised a novel member of the PDE7 family of cyclic nucleotide phosphodiesterases (PDE), which we have designated PDE7B. Mouse and human full-length cDNAs were isolated encoding a protein of 446 and 450 amino acids, respectively. The predicted protein sequence of PDE7B showed highest homology (70% identity) to that of PDE7A. Northern blot analysis identified a single 5.5-kb transcript with highest levels detected in brain, heart, and liver. Kinetic analysis of the mouse and human purified recombinant enzymes show them to specifically hydrolyse cAMP with a K_m of 0.1 and 0.2 μM respectively. Inhibitor studies show sensitivity to dipyridamole, IC_{50} of 0.51 and 1.94 μM, and IBMX, IC_{50} of 3.81 and 7.37 μM, for the mouse and human enzymes, respectively. This shows that dipyridamole is not selective for cGMP over cAMP PDEs as previously believed. Other standard PDE inhibitors including zaprinast, rolipram, and milrinone do not significantly inhibit PDE7B.

Key Words: **phosphodiesterase; cAMP; dipyridamole; IBMX; PDE7B.**

The cyclic nucleotides cAMP and cGMP are intracellular second messengers for a wide range of extracellular signals such as neurotransmitters, hormones and light. The responses evoked by these stimuli are mediated by the action of cyclic nucleotides on their intracellular targets, such as kinases and ion channels. 3′, 5′-Cyclic nucleotide phosphodiesterases (PDEs) are a diverse family of enzymes (1) which play an important role in signal transduction (2–4) by regulating the intracellular concentrations of cAMP and cGMP by hydrolysing them to biologically inactive nucleoside-5′-monophosphates. Ten biochemically distinct gene subfamilies of mammalian PDEs have been identified based on amino acid sequence, substrate specificity, inhibitor sensitivity and allosteric cofactors (5–9). The sub-families are: PDE1, Ca^{2+}/calmodulin-dependent; PDE2, cGMP-stimulated; PDE3, cGMP-inhibited cAMP-hydrolysing; PDE4, cAMP-specific, rolipram sensitive; PDE5, cGMP-specific; PDE6, photoreceptor cGMP-specific; PDE7, cAMP-specific, rolipram insensitive; PDE8, cAMP-specific IBMX insensitive; PDE9, cGMP-specific IBMX insensitive; PDE10 dual substrate, IBMX sensitive. PDEs contain a conserved C-terminal catalytic domain of ~270 amino acids (10) and an N-terminal domain involved in regulating catalytic activity by binding cofactors, and in specifying subcellular localisation (11). The majority of PDE families contain multiple isozymes coded for by distinct genes and frequently multiple splice variants are associated with each isozyme (1). Within families there is at least 70–80% sequence similarity, whereas between families there is less than 50% similarity (1). PDE family members can exhibit distinct expression profiles and this provides the potential for exquisite, tissue specific regulation of cyclic nucleotide levels. In order to increase our understanding of the complex mechanisms of cyclic nucleotide signal transduction regulation, we have searched for novel cyclic nucleotide phosphodiesterases.

We report here the cloning, tissue distribution, expression and biochemical characterisation of the mouse and human PDE7B. The cDNAs encoding this gene were isolated from 13.5 day mouse embryo and human foetal brain cDNA libraries, respectively. Functional expression in baculovirus shows PDE7B to spe-

The sequences reported in this paper have been deposited in the EMBL database (Accession Nos. AJ251859 and AJ251860).

Abbreviations used: PDE, phosphodiesterase; cAMP, adenosine 3′5′-cyclic monophosphate; cGMP, guanosine 3′5′-cyclic monophosphate; EST, expressed sequence tag; kb, kilobase pair; bp, base pair; ORF, open reading frame; UTR, untranslated region; IBMX, 3-isobutyl-1-methyl-xanthine; PAGE, polyacrylamide gel electrophoresis; IMAGE, Integrated Molecular Analysis of Genomes and their Expression database.

[1] To whom correspondence should be addressed. Fax: 44-1304-655592. E-mail: Mark_Fidock@sandwich.pfizer.com.

0006-291X/00 $35.00

cifically hydrolyse cAMP and sequence comparisons with known PDEs, together with its kinetic profile and inhibitor sensitivities, show that this enzyme is an additional member of the PDE7 family.

EXPERIMENTAL PROCEDURES

Database searching for PDE EST sequences. The full-length coding sequences of all the published mammalian PDEs were used as a query to search the I.M.A.G.E. database of expressed sequence tags (ESTs) (12) using the Basic Local Alignment Search Tool (BLAST) (13). Detailed bioinformatic analysis of the search results indicated that IMAGE clone 1364394 may encode a fragment of a novel PDE which we later named PDE7B (5). IMAGE clone 1364394 was obtained from Research Genetics (Huntsville, AL).

Cloning of full-length PDE7B cDNA sequences using GeneTrapper. The mouse PDE7A2 (Accession No. U68171) and IMAGE clone 1364394 nucleotide sequences were aligned using the Clustalw program (14). A region of 30 nucleotides at position 320–291 in clone 1364394 were chosen since these residues are specific for this EST. A capture oligonucleotide 5′-GGT CAC AGA ACT GCC ACT ATG GTT AAA TGT-3′ was synthesised by LTI (Life Technologies Ltd., Paisley, Scotland) and PAGE purified. The oligonucleotide was biotinylated using terminal transferase incorporating 14-dCTP as described in the GeneTrapper Manual (LTI, Catalogue number 10356-020). Mouse 13.5 day embryo and a human foetal brain cDNA libraries were purchased from LTI. 100 ml of Luria Broth (10 g tryptone, 5 g yeast extract, 5 g NaCl/litre) + 100 ug/ml Ampicillin was inoculated with 2.5×10^9 c.f.u of each cDNA library. Each library was grown up and prepared individually. The cultures were grown up overnight at 30°C and plasmid DNA was prepared as described in the GeneTrapper protocol (LTI). Detailed methods for the positive selection of cDNA clones using GeneTrapper (LTI) are outlined in the kit protocols, these were followed except for the following modifications. The single stranded cDNA library was combined with 20 ng of biotinylated capture oligonucleotide and hybridised at 37°C for 1 h. The eluted single stranded DNA was repaired using the capture oligonucleotide as a primer for DNA polymerase extension. The repaired cDNA libraries were electroporated in to the *E. coli* strain DH10B (LTI) using the manufacturer's protocol.

Identification of PDE7B. The electroporated DH10B *E. coli* containing the selected cDNA libraries were plated out on Luria Broth agar plates (5 g Tryptone, 10 g yeast extract, 5 g NaCl, 14 g Agar/litre) plus 100 μg/ml Ampicillin and grown overnight at 37°C. Twenty-four colonies were picked into Luria Broth and grown to saturation overnight at 37°C. Plasmid DNA was isolated using Qiagen miniprep kits (Qiagen Ltd., Crawley, West Sussex) and positive clones were identified by PCR screening using primers specific for clone 1364394, sense 5′ GAT GCA TGG AAG TAT CTT TGG 3′ and antisense 5′ CAG AGT GTC AAA TGT GTT TGC 3′. DNA digestion using *SalI* and *NotI* restriction endonucleases were used to identify insert size.

Analysis of DNA sequences. DNA sequencing was performed on both strands by fluorescence-tagged dye terminator cycle sequencing (Perkin-Elmer, Norwalk, CT) followed by analysis on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were analysed by BLAST (Basic Local Alignment Search Tool) searching (13).

Northern blot and RNA dot blot analysis. Mouse multiple tissue Northern blots and human RNA dot blots were purchased (Clontech UK Ltd, Basingstoke, Hampshire, Cat. No. 7770-1) and prehybridised in ExpressHyb hybridization solution (Clontech) for 1 h at 55°C. DNA probes were generated from the full-length mouse and human PDE7B sequences using the Megaprime Random Labelling System (Amersham, St. Albans, Hertfordshire) according to the manufacturer's instructions. The DNA was labelled with 50 μCi of [α-^{32}P]dCTP (Amersham, Cat. No. AA0005). The labelled probes were then added to fresh ExpressHyb solution and left to hybridise to the blots overnight at 55°C with gentle shaking at 50 rpm. Blots were then washed 3 times at room temperature for 10 min each in 2× SSC (150 mM NaCl, 30 mM Na citrate)/0.1% SDS followed by two washes at 55°C for 20 min each in 0.2× SSC (15 mM NaCl, 3 mM Na Citrate)/0.1% SDS. Blots were exposed to X-Omat AR autoradiographic film (Kodak, Luton, Bedfordshire) for 48 h prior to development and subsequently stripped using standard protocol (15) and re-probed with β-actin to confirm equal loading of the RNA samples.

Expression of PDE7B in insect cells. The full-length coding regions of mouse and human PDE7B were amplified using the following primers: Human forward primer 5′-CACTCTAGAACCATGTCTTGTTTAATGG-3′ (bp −12 to 16) and reverse primer 3′-GCTGTCGGGGATCACTGGCCGAGCTCAATCTG-5′ (bp 1335 to 1366), mouse forward primer 5′-CACTCTAGAACCATGTCTTGTTTAATGG-3′ (bp −12 to 16) and reverse primer 3′-CGGTGCGGGATTACTTCGAGCTCC-5′ (bp 1326 to 1349). The primers were designed to incorporate an *XbaI* site at the 5′ end and an *XhoI* site at the 3′ end of the PCR fragments for cloning purposes. The two PCR products were subcloned into pCR2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Groningen, Netherlands, Cat. No. K4500-01) according to manufacturer's instructions and sequence verified. The full-length coding sequence was then subcloned into the baculovirus transfer vector pFASTBAC 5′-FLAG between the *XbaI* and *XhoI* sites within the multiple cloning site. The pFASTBAC (LTI) vector had been modified to include an N-terminal nine amino acid FLAG tag for use in detection and purification hence the N-terminus of the PDE7B protein would begin with the sequence MDYKDDDDK. Recombinant virus stocks were prepared according to the manufacturer's protocol (LTI) and viral titres determined by plaque assay. SF-9 cells were cultured in SF900II serum free media (LTI) at 27°C. To obtain recombinant protein, 50 ml cultures containing 2.5×10^7 cells were infected at a multiplicity of infection of 0.5. Cells were harvested 72 h post infection.

Preparation of SF9 cell crude lysates. Transfected cells were harvested by centrifugation and resuspended to a concentration of 1×10^7 cells/ml in chilled homogenisation buffer (20 mM HEPES pH 7.2, 1 mM EDTA, 250 mM sucrose, 1 complete protease inhibitor cocktail tablet (Boehringer Mannheim UK, Lewes, East Sussex, Cat No. 1 697 498) per 50 ml buffer). The cells were disrupted by sonication on ice and the cell debris removed by centrifugation at 14,000g for 15 minutes at 4°C. The supernatant was removed and snap frozen before being stored at −80°C.

Purification of PDE7B enzyme. The crude lysates containing the PDE7B-FLAG fusion protein were passed down a FLAG antibody M2 affinity column (Sigma-Aldrich, Poole, Dorset Cat No. A1205) according to manufacturer's protocol. Purified protein was eluted under conditions as specified by the manufacturer of the affinity beads, split into aliquots, flash frozen in liquid nitrogen and stored at −80°C.

Kinetics and inhibitor studies on affinity purified protein. Phosphodiesterase activity of the mouse and human PDE7B was measured using the phosphodiesterase Scintillation Proximity Assay (SPA) (Amersham) according to the manufacturer's protocol with the following modifications: all assays were done in triplicate in 96 well format. Reaction times and enzyme dilution were optimised so that the lowest substrate concentration gave no more than 30% conversion of substrate to product to ensure linearity. The reactions contained 25 μl of the appropriately diluted enzyme, 25 μl buffer (20 mM Tris with 5 mM MgCL2.6H2O, pH 7.4 plus 2 mg/ml BSA) and initiated by the addition of 50 μl of either cAMP or cGMP to give a total reaction volume of 100 μl. [^{3}H]-cAMP (Amersham Cat. No. TRK304 B70, 24.Ci/mmol) or [^{3}H]-cGMP (Amersham Cat. No. TRK392 B37, 10.7 Ci/mmol) was mixed with the corresponding cold cyclic nucleotide to give a final concentration range of 1 μM–0.002 μM. This was achieved by performing doubling dilutions across a 96 well plate. Following a 40 min incubation at 30°C, the plates were

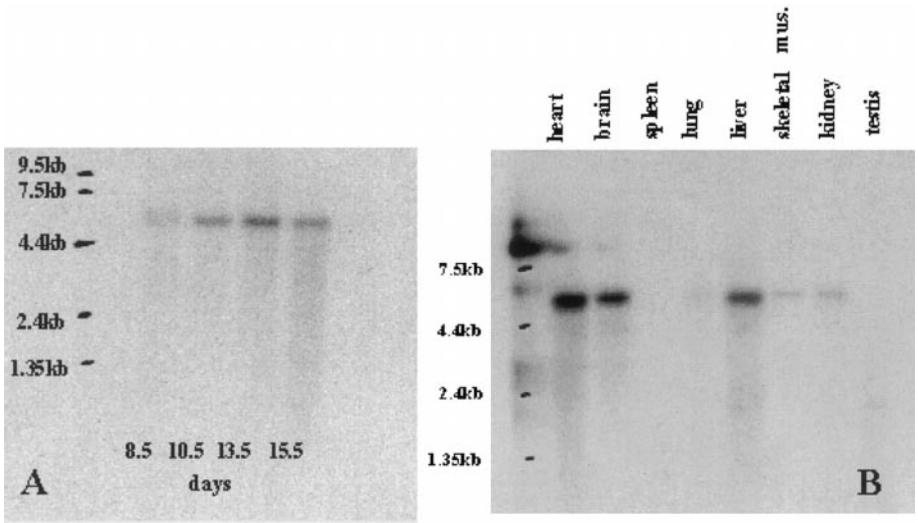

FIG. 1. Northern blot analysis of mouse PDE7B expression. Each lane of the two blots contains 2 μg of mouse poly(A)$^+$ RNA. A transcript of 5.5 kb was highly expressed in mouse embryo day 13.5 (A), and in adult heart, brain and liver (B). Blots were stripped and reprobed with β-actin to show equal loading of RNA samples (data not shown).

immediately centrifuged at 2000 rpm for 5 min and then counted on TopCount. Background levels for each cAMP concentration were determined using a Scintillation Counter. Average counts of triplicate results for each assay were determined and the corresponding background subtracted. Counts per min for each assay were converted into pmol of cAMP hydrolysed per min per ml of enzyme and plotted against cAMP concentration (μM). For inhibitor profiling a concentration range of 0.5–300 μM in 1% dimethyl sulphoxide for each inhibitor was used and cAMP concentration was kept constant at 1/3 K_m. The assay blank contained all reagents minus the enzyme. Values for K_m and IC_{50} were determined using the computer package GraFit4.

RESULTS

The identification of mouse PDE7B. PDE7B partial cDNA sequence was initially identified by bioinformatic screening of the IMAGE EST database. Full-length sequencing revealed that IMAGE clone 1364394, isolated from mammary gland, contained an insert of 725 bp. Database searches using BLAST showed that the DNA insert was most similar to PDE7A (40%) and did not have a high level of homology to any known PDE, indicating that the clone encoded a fragment of a novel PDE. Further analysis of this sequence revealed that clone 1364394 was incomplete but it did contain a putative ORF extending from an ATG initiator through to residue 98 of the sequence.

Cloning, tissue distribution and sequence analysis of the mouse and human PDE7B. To facilitate the isolation of a full-length cDNA, the expression profile of PDE7B was determined in a range of mouse tissues using Northern hybridisation (Fig. 1). This identified a transcript of 5.5 kb which was particularly highly expressed in the mouse embryo at 13.5 days and in the heart, brain and liver of the adult mouse. This transcript is also present at low levels in 8.5, 10.5, 15.5 day embryos and in skeletal muscle, kidney and lung of the adult. A transcript was not detected in the spleen or testis. Based on these data a 13.5 day embryo cDNA library (LTI) was used as a template to isolate the full-length sequence using a positive cDNA selection

FIG. 2. RNA master blot analysis of human PDE7B expression. Dot blot contains normalised loading of each poly(A)$^+$ RNA from 50 different human tissues. mRNA for human PDE7B was most highly expressed in caudate nucleus, putamen, and occipital lobe of the brain, heart, liver, ovary, pituitary gland, kidney, small intestine, and thymus. Blots were stripped and reprobed with β-actin to show equal loading of RNA samples (data not shown).

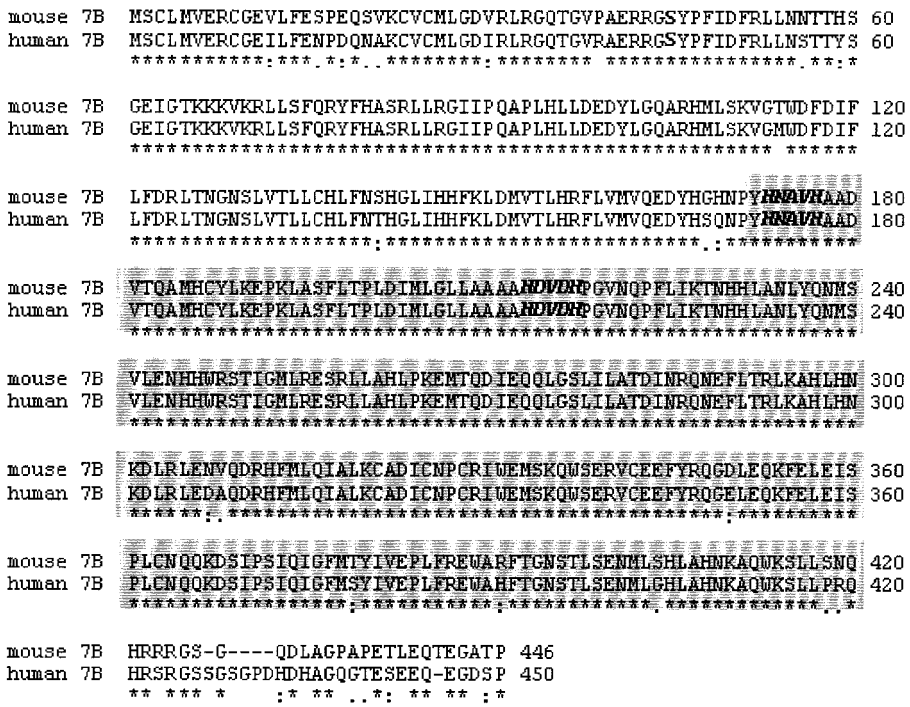

FIG. 3. Alignment of the amino acid sequence of mouse and human PDE7B. The single cAMP-dependent protein kinase phosphorylation site at serine 45 is shown in bold type. The two putative divalent cation binding motifs are shown in italics. The grey boxed region outlines the catalytic domain. A "*" indicates identity, ":" indicates where one of the strong groups is fully conserved, and "." indicates where one of the weaker groups is fully conserved (15). Mouse and human sequences have been deposited in the EMBL database with Accession Nos. AJ251858 and AJ251860, respectively.

technique, GeneTrapper (LTI). This was achieved by using a PDE7B specific oligonucleotide designed using the observed alignment between PDE7A2 and clone 1364394. PCR carried out using primers specific for Clone 1364394, identified cDNAs that contained at least the EST fragment. Restriction endonuclease digestion identified a number of clones containing an insert of 2725 bp and full sequence analysis showed that these clones encoded an ORF of 446 amino acids with a predicted molecular mass of 51.3 kDa. Further analysis of the region directly upstream to the start methionine identifies stop codons in all three reading frames indicating the full N-terminal coding sequence is present.

In parallel, the full-length human cDNA was isolated by GeneTrapper using the same specific capture oligonucleotide and a human foetal brain cDNA library as template. A number of clones were isolated containing an insert of 2653 bp and full sequence analysis identified an ORF of 450 residues with a predicted molecular mass of 51.8 kDa. Stop codons in all three reading frames indicating the full N-terminal coding sequence is present, were identified in the region directly upstream to the start methionine. Using the full-length human cDNA an RNA master blot was probed (Fig. 2). This identified that the mRNA for the human PDE7B is highly expressed in the caudate nucleus, putamen and occipital lobe of the brain and peripherally in the heart, ovary, pituitary gland, kidney, liver, small intestine and thymus. Lower levels were observed in skeletal muscle, colon, bladder, uterus, prostate, stomach, adrenal gland, and thyroid gland. The transcript was not detected in testis, spleen, pancreas, peripheral leukocytes, lymph node, bone marrow, aorta or cerebellum.

Sequence comparison of the mouse and human PDE7B shows that they share 91% amino acid identity and both cDNAs contain a cAMP dependent protein kinase phosphorlation site at serine 45. The predicted human ORF is extended by 4 amino acids, this is the result of insertions at positions 427, 429–432 and the deletion of a single amino acid at position 441 of the mouse sequence (Fig. 3). A phylogenetic alignment of the 230 amino acid catalytic domain of PDE7B (amino acids 172–420) with representatives of other PDEs shows that PDE7B has highest homology to and clusters with PDE7A (70% identity) (Fig. 4). Further bioinformatic analysis shows that this domain shares less than 50% sequence identity with the equivalent regions in the other nine known PDE sub-families and thus confirms PDE7B's designation as the second representative of the PDE7 family.

The PDE7B sequence contains two putative divalent cation binding motifs $HXXXHX_{8-20}D$ (amino acids 173–

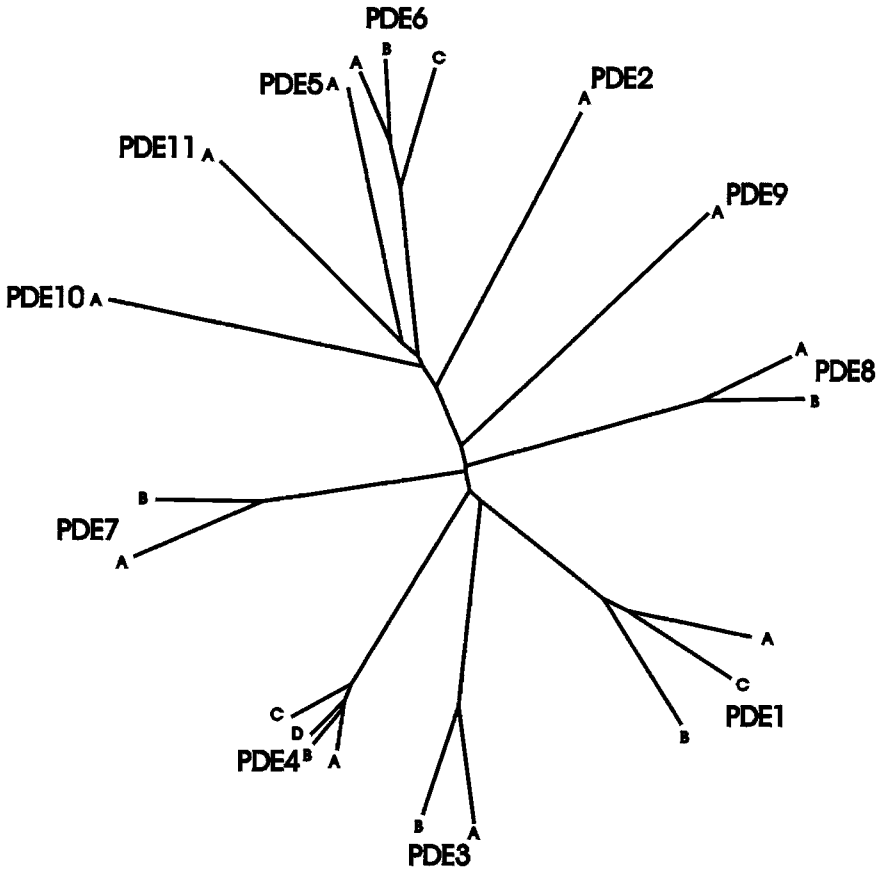

FIG. 4. Radial phylogenetic tree. A radial phylogenetic tree for the PDE gene family that shows PDE7B to be most similar to PDE7A. A multiple alignment consisting of the human PDE catalytic domains and a distance matrix of similarity scores as derived by the Neighbour-joining method using Clustalw and Phylip packages (15).

180, 213–270), which have been shown previously to be essential for hydrolytic activity (16, 17). This strongly supports the hypothesis that PDE7B encodes a functional phosphodiesterase enzyme.

Expression of mouse and human PDE7B in insect cells. Full-length PDE7B was expressed using the baculovirus expression system (LTI). A fusion protein was generated containing an N-terminal 9 amino acid FLAG tag to facilitate detection and purification. Western blotting of lysates made from the infected Sf-9 cells showed a polypeptide with an apparent molecular mass of ~52 kDa which is detected by an anti-FLAG antibody (data not shown). This is in agreement with the 52 kDa predicted for FLAG-PDE7B. This polypeptide was not observed in mock transfected cells. Infected cell lysates exhibited 100 fold greater cAMP hydrolysing activity than mock transfected cells showing recombinant PDE7B to be catalytically active against cAMP. This activity could be purified with anti-FLAG affinity gel indicating that cAMP hydrolysis was specifically associated with the FLAG-PDE7B fusion protein. FLAG affinity purified PDE7B was used for kinetic analysis. Coomassie staining of the purified fractions showed the protein to be 80% pure.

Kinetic analysis of mouse and human PDE7B. To determine the K_m of the enzyme, the rate of hydrolysis of cAMP and cGMP was measured at a variety of substrate concentrations using a diluted enzyme preparation (as described in Experimental Procedures). Figure 5 shows the reaction velocity as a function of the substrate concentration for mouse and human PDE7B. Both enzymes exhibited standard Michaelis-Menton kinetics and showed a high affinity for cAMP with K_m values of 0.1 and 0.2 μM, for mouse and human respectively. Furthermore, PDE7B did not hydrolyse cGMP (>10 μM) under similar conditions of enzyme concentration and incubation time. The effects of a variety of standard PDE inhibitors on PDE7B were examined. IC_{50} was defined as the concentration of inhibitor that reduced the rate of substrate turnover to 50% of the uninhibited control level. Control reactions using enzyme alone $\pm$1% DMSO showed that DMSO at this concentration had no effect on enzyme activity. Studies carried out on mouse and human purified enzyme us-

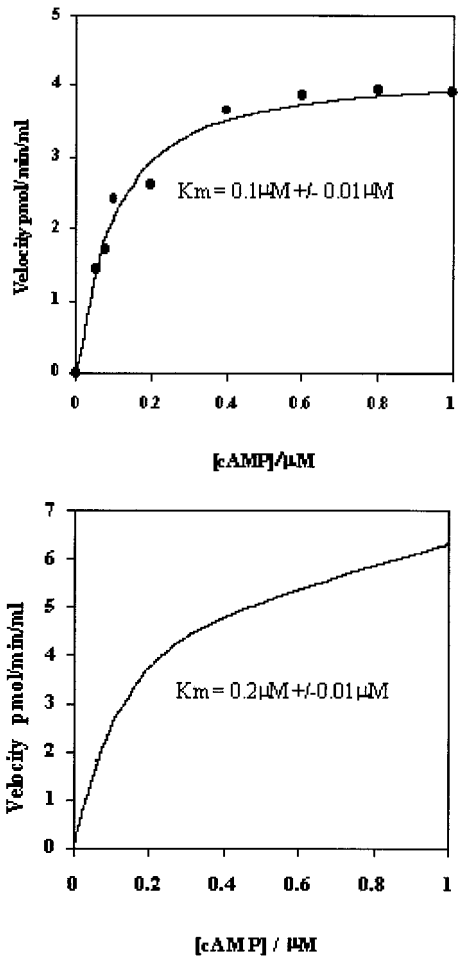

FIG. 5. Mouse and Human PDE7B kinetics. The plots show reaction velocity in pmol/min/ml against cAMP concentration (μM). K_m values are shown as mean ± SEM (n = 3 separate experiments). Details of methodology are given under Experimental Procedures.

ing a range of standard PDE inhibitors showed dipyridamole to be the most potent, IC_{50} values 0.51 and 1.94 μM respectively, while IBMX was also active, IC_{50} values 3.81 and 7.37 μM respectively. Zaprinast, rolipram, and milrinone were inactive up to 100 μM (Table I).

DISCUSSION

Multiple cyclic nucleotide phosphodiesterase families provide the potential for complex regulation of cyclic nucleotide levels that, in turn, mediate signal transduction. Elucidation of all such phosphodiesterases will allow a greater understanding of their function in specific signalling pathways, their relevance to disease pathology and, ultimately, their potential as therapeutic targets.

We have cloned and characterised both the mouse and human PDE7B. These genes show high sequence identity (91%) by amino acid alignment, suggesting these are species orthologues. Like all mammalian phosphodiesterases sequenced to date, PDE7B contains a conserved catalytic domain sequence of approximately 270 amino acids in the C-terminal half of the protein that is known to be essential for catalytic activity. This segment exhibits 70% sequence identity to PDE7A and 33–46% sequence identity to the corresponding domains of the other known PDEs. Furthermore, the putative N-terminal regulatory domain of PDE7B contains a phosphorylation site as predicted by the PROSITE program (18) (Fig. 3). This phosphorylation site is conserved across the PDE7 family and although the significance of this observation has not been tested, it may provide the potential for allosteric regulation (19). This, together with the sequence analysis, confirms PDE7B as a new member of the PDE7 family.

Northern blot studies show the presence of a 5.5 kb mRNA transcript in a variety of tissues. Interestingly, the tissue distribution is largely conserved between mouse and human, suggesting the physiological role of PDE7B may also be conserved. We have observed a size difference between the mRNA transcript and the cloned cDNA (~2.5 kb). Sequence analysis has shown the cDNAs to encode a full-length polypeptide, therefore, the most likely explanation for the observed difference in size is the presence of an extended 3′ untranslated region. This suggestion is supported by the observation that neither the mouse nor human cDNAs contain a classical polyadenylation signal in the 3′ UTR (20).

TABLE I

IC_{50} Values for Inhibitors of Mouse and Human PDE7B by a Range of Standard PDE Inhibitors

Inhibitor	Selectivity (IC_{50})	IC_{50} for murine PDE7B (μM)	IC_{50} for human PDE7B (μM)
Dipyridamole	PDE5/6/8 (0.9/0.38/1.5 μM)	0.51 ± 0.06	1.94 ± 0.26
IBMX	non-selective (2–50 μM)	3.81 ± 0.42	7.37 ± 0.89
Milrinone	PDE3/4 (3.2 μM/19 μM)	>100	>100
Rolipram	PDE4 (2.0 μM)	>100	>100
Zaprinast	PDE5/6 (0.76 μM/0.15 μM)	>100	>100

Note. Compounds were tested between the ranges 0.5–300 μM. cAMP concentration was kept constant at $\frac{1}{3} K_m$ for each enzyme. IC_{50} values are shown as mean ± SEM (n = 3 experiments). Details of methodology are given under Experimental Procedures.

Kinetic analysis shows the recombinant PDE7B protein to be comparable to PDE7A; both are cAMP specific hydrolysing enzymes with a high affinity for cAMP (21). Sensitivity to the PDE inhibitors dipyridamole and IBMX has been demonstrated, whilst no significant inhibition by a number of other standard PDE inhibitors was observed. The compound which showed the greatest inhibition of PDE7B was dipyridamole (IC_{50} values 0.51 and 1.94 μM for mouse and human respectively). This inhibitor is generally considered to be relatively specific for cGMP selective PDEs, however, others have shown that this compound inhibits cAMP selective PDEs (7) and we have now shown that it is a relatively potent inhibitor of PDE7B. In future, this inhibitor should be considered as non-selective. Furthermore, PDE7B has been shown to demonstrate a unique inhibitor profile compared to PDE7A and such differences would be favourable for the discovery of substrate competitive inhibitors specific for PDE7B (22).

In summary we have identified a novel mouse and human PDE with high specificity for cAMP. Sequence analysis, kinetic and inhibitor profiles confirm its designation as a new member of the PDE7 family, PDE7B. The biological role of PDE7B is not well established, but selective PDE7B inhibitors may be useful in better defining which cAMP driven process is regulated by this enzyme.

ACKNOWLEDGMENTS

The authors thank Dr. Ian Harrow for providing the phylogenetic tree analysis and Drs. Geoff Johnston and Stephen Ballard for helpful comments on this manuscript.

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