Novel alternative splice variants of rat phosphodiesterase 7B showing unique tissue-specific expression and phosphorylation

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cDNA species coding for novel variants of cyclic-AMP-specific phosphodiesterases (PDEs), namely the PDE7B family, were isolated from rats and characterized. Rat PDE7B1 (RNPDE7B1) was composed of 446 amino acid residues. Rat PDE7B2 (RNPDE7B2) and PDE7B3 (RNPDE7B3), which possessed unique N-terminal sequences, consisted of 359 and 459 residues respectively. Northern hybridization analysis showed that rat PDE7B transcripts were particularly abundant in the striatum and testis. PCR analyses revealed that rat PDE7B2 transcripts were restricted to the testis and that low levels of PDE7B3 transcripts were expressed in the heart, lung and skeletal muscle. *In situ* hybridization analysis demonstrated that rat PDE7B transcripts were expressed in striatal neurons and spermatocytes. In spermatocytes, rat PDE7B transcripts were expressed in a stage-specific manner during spermatogenesis. The K_m values of

recombinant rat PDE7B1, PDE7B2 and PDE7B3 for cAMP were 0.05, 0.07 and 0.05 μ M respectively. Each rat PDE7B variant was the most sensitive to 3-isobutyl-1-methylxanthine (IC_{50} 1.5–2.1 μ M). Two phosphorylation sites for cAMP-dependent protein kinase (PKA) were found in rat PDE7B1 and PDE7B3, whereas rat PDE7B2 possessed one site. PKA-dependent phosphorylation was observed in C-terminal phosphorylation sites of three rat PDE7B variants, in addition to unique N-terminal regions of rat PDE7B1 and PDE7B3. Unique tissue distribution and PKA-dependent phosphorylation of PDE7B variants suggested that each variant has a specific role for cellular functions via cAMP signalling in various tissues.

Key words: brain, *in situ* hybridization, phosphodiesterase, protein kinase A, testis.

INTRODUCTION

Second messengers such as cAMP and cGMP, which regulate many cellular functions, are produced in response to extracellular stimulation and are metabolized by 3',5'-cyclic-nucleotide phosphodiesterases (PDEs) [1,2]. Various physiological roles of PDEs such as phototransduction [3], contraction of smooth muscle [4], female fertility [5], insulin secretion [6] and activation of T cells [7] have been reported. Genes encoding PDE enzymes are classified into 11 families on the basis of amino-acid sequence similarity, biochemical properties and inhibitor profiles [1,8]. Furthermore, some families consist of subfamilies and a large number of alternative splice variants [1,8,9]. In most instances these PDE enzymes show distinct expression patterns in tissues and different subcellular localizations [1,8,9]. One example of unique tissue-specific expression is PDE11A: PDE11A3 and PDE11A4 transcripts are expressed specifically in the testis and prostate respectively, as we have described previously [10]. With regard to subcellular localization, PDE7A1 protein is present in both the soluble and particulate fractions of many tissues, whereas PDE7A2 protein exists only in particulate fractions of fetal heart and skeletal muscle [11]. It has been also reported that distinct alternative splice variants exhibit different regulation in response to some kinds of kinases [9,12,13] and associated proteins [14]. Extracellular signal-regulated protein kinase 2 (ERK2)-dependent phosphorylation of Ser⁵⁷⁹ of PDE4D3 is known to cause inhibition of this enzyme, whereas subsequent phosphorylation of Ser⁵⁴ by cAMP-dependent protein kinase (PKA) serves

to ablate the ERK2-induced inhibition of PDE4D3 [15]. Such unique tissue expression, regulation and subcellular localization of alternative splice variants of PDEs suggest that each variant is closely related to the specific regulation of functions in tissues and the compartmentalization of cyclic-nucleotide signalling in cells. The identification of splice variants of PDEs is now needed for a further understanding of cyclic nucleotide metabolism.

We have reported the identification and characterization of a human cAMP-specific PDE, PDE7B [16]. PDE7B transcripts are particularly abundant in the putamen and caudate nucleus in humans. Mouse and human PDE7B [17] and PDE7A have putative phosphorylation sites for PKA. However, no investigation has been made into whether both PDE7A and PDE7B are phosphorylated by PKA.

Here we report the identification of PDE7B1 and two novel alternative splice variants, PDE7B2 and PDE7B3, in rats. We also show tissue-specific expression, enzymic properties and PKA-dependent phosphorylation of these splice variants. The findings provide important information for studies on cAMP metabolism.

MATERIALS AND METHODS

Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Shuzo (Kyoto, Japan). [α -³²P]dCTP, [γ -³²P]ATP, [³H]cAMP and Hybond-N⁺ nylon membrane were purchased from Amersham Biosciences. The TA-cloning vector

Abbreviations used: ERK2, extracellular signal-regulated protein kinase 2; IBMX, 3-isobutyl-1-methylxanthine; ORF, open reading frame; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; RACE, rapid amplification of cDNA ends.

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pGEM-T Easy was from Promega. The mammalian expression vector pFLAG-CMV-2 (in which CMV stands for cytomegalovirus) was obtained from Eastman Kodak. The monoclonal antibody M5 against FLAG (MDYKDDDK; single-letter amino acid codes), erythro-9-(2-hydroxy-3-nonyl)-adenine, milrinone and dipyridamole were purchased from Sigma. Rat multiple tissue northern (MTN) blots, Marathon-Ready cDNA (rat testis and skeletal muscle) and rat multiple tissue cDNA (MTC) panel I were obtained from ClonTech. Digoxigenin RNA-labelling kit and digoxigenin nucleic acid detection kit were purchased from Roche Diagnostics. 3-Isobutyl-1-methylxanthine (IBMX) was from Wako Pure Chemicals (Osaka, Japan). SCH51866 was synthesized by Tanabe Seiyaku Co. (Osaka, Japan).

General methods

Nucleotide sequences were determined with an automated DNA sequencer ABI Prism 310 and a BigDye terminator cycle sequencing reaction kit (PE Applied Biosystems). The computer program Genetyx (Software Development, Japan) was used to analyse nucleotide and amino acid sequence data.

Amplification of rat PDE7B1 cDNA

For the isolation of rat PDE7B, PCR was performed with rat brain cDNA included in rat MTC panel I and the primer set 5'-GTTGCTGGATTCTGCAGCAC-3' and 5'-AGGAGCTACTGCCAGTCAGCGTGACC-3'. These primers were designed on the basis of the nucleotide sequences of human and mouse PDE7B cDNA species. PCR was performed through 33 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s. An amplified fragment was subcloned into pGEM-T Easy, then sequenced.

Northern blot analysis

Northern blot analysis was performed with a rat MTN blot. To examine the expression of PDE7B transcripts in rat brain, various regions of the brain of a male Sprague–Dawley rat at 10 weeks were excised, and total RNA species were isolated by using Isogen (Nippon Gene, Toyama, Japan). Poly(A)⁺ RNA species were purified from total RNA with an mRNA separator kit (ClonTech). Purified Poly(A)⁺ RNA species (2 µg) were subjected to electrophoresis in 1% (w/v) agarose/0.66 M formaldehyde gel and transferred to Hybond-N⁺ membrane. All blots were hybridized with the ³²P-labelled probe (nt 67–443 of rat PDE7B1 cDNA) and hybridization was performed in ExpressHyb solution (ClonTech) in accordance with the manufacturer's instructions. After hybridization, blots were finally washed in 0.1 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 50 °C, and then exposed to X-ray film with an intensifying screen at –70 °C for 2 weeks.

Experiments with 5' rapid amplification of cDNA ends (RACE)

For isolation of rat PDE7B2 cDNA, 5' RACE was performed with a Marathon-Ready cDNA library (rat testis) and the primer set 5'-CCATCCTAATACGACTCACTATAGGGC-3' (AP1, supplied in the product) and 5'-GAGTTGAAGAGGTGACA-CAACAGAGTTACCAGACTG-3' (nt 350–385 of rat PDE7B2 cDNA). All 5' RACE experiments were conducted in accordance with the manufacturer's instructions. RACE products were subcloned into pGEM-T Easy, then sequenced. To verify the presence of the transcripts coding for a full-length open reading frame (ORF) of rat PDE7B2, PCR was performed with a rat testis cDNA library and the primer set 5'-CTCCACATTGAC-

TCCTGGGAGC-3' and 5'-CGGACAGATGGCCGTGCTCC-3'. The PCR product was subcloned and the sequence was confirmed. Isolation of the cDNA encoding rat PDE7B3 was achieved by PCR with a Marathon-Ready cDNA library (rat skeletal muscle) and the primer set 5'-GTCACAGAACTGCC-ACTATG-3' and 5'-CGGACAGATGGCCGTGCTCC-3'. The PCR product of the appropriate size was subcloned into pGEM-T Easy, then sequenced, resulting in pG-R7B3N1.

PCR analysis

To detect the mRNA species encoding PDE7B variants, PCR was performed with rat MTC panel I and the following primer sets: 5'-ATGGTTGAGAGGTGTGGCGAAGTC-3' and 5'-GTCCCACATTCCAACCTTTGGAGAGC-3' for PDE7B1 and PDE7B3; 5'-CTCCACATTGACTCCTGGGAGC-3' and 5'-GTCCCACATTCCAACCTTTGGAGAGC-3' for PDE7B2. PCR was performed for 33 cycles for PDE7B1 and PDE7B3 or 30 cycles for PDE7B2, as follows: denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min. The PCR products were subjected to non-denaturing 5.5% (w/v) PAGE. After subcloning, the products were sequenced and confirmed to be part of rat PDE7B1, PDE7B2 and PDE7B3 cDNA species.

In situ hybridization analysis

Fragments coding for rat PDE7B1 (nt 67–443) and rat PDE7B2 (nt 10–310) were obtained by PCR with the following primer sets: 5'-AAAGGTCACAGAACTGCCAGTATGG-3' and 5'-GTCCCACATTCCAACCTTTGGAGAGC-3'; 5'-CTCCACATTGACTCCTGGGAGC-3' and 5'-GTCCCACATTCCAACCTTTGGAGAGC-3'. Specifically amplified products were subcloned into pGEM-T Easy, producing pGEM-r7B1 and pGEM-r7B2 respectively. The plasmids pGEM-r7B1 and pGEM-r7B2 were linearized with *Aat*II for the anti-sense probe and *Pst*I for the sense probe. RNA riboprobes for the *in situ* hybridization of rat PDE7B mRNA species were prepared with a digoxigenin RNA-labelling kit in accordance with the manufacturer's instructions. The anti-sense and sense probes were synthesized from 1 µg of the linearized plasmid templates with SP6 and T7 polymerases respectively. Whole brains were excised from male Sprague–Dawley rats at 10 weeks, frozen in solid CO₂ and stored at –70 °C until use. After frozen sections (10 µm thick) had been mounted on silane-coated slides, they were soaked with 4% (v/v) formaldehyde. A testis from a male Sprague–Dawley rat at 10 weeks was fixed in fixation solution [4% (v/v) paraformaldehyde/0.2 M sodium phosphate/10 mM picric acid], dehydrated in ethanol, then embedded in paraffin. Sections of the testis (3 µm) were deparaffinized and rehydrated. All sections were treated with 14 µg/ml proteinase K/0.1 M triethanolamine/0.25% (v/v) anhydrous acetic acid followed by dehydration in a graded ethanol series. After prehybridization in hybridization solution [20 mM Tris/HCl (pH 8.0)/300 mM NaCl/10% (v/v) sodium dextran sulphate/50% (v/v) formamide/0.2% *N*-lauroylsarcosine/100 µg/ml salmon-sperm DNA/1 × Denhardt's solution] at 50 °C for 30 min, hybridization was performed with digoxigenin-labelled riboprobes in hybridization solution at 50 °C for 16 h in a humid chamber. The sections were washed with a high-stringency buffer [50% (v/v) formamide/300 mM NaCl/30 mM sodium citrate] at 60 °C for 30 min, treated with RNaseA (1 µg/ml) at 37 °C for 10 min, then washed with the high-stringency buffer. They were incubated with anti-digoxigenin polyclonal antibody diluted in buffer A [100 mM Tris/HCl (pH 7.5)/150 mM NaCl] containing 1.5% (v/v) blocking reagent (Roche Diagnostics) at 25 °C for 5 h, then

washed extensively with buffer A. The sections were incubated with a freshly prepared colour-substrate solution [100 mM Tris/HCl (pH 9.5)/100 mM NaCl/50 mM MgCl₂] containing Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate; colour was developed for 2 days in the absence of light at room temperature.

Construction of expression plasmids

The DNA fragment coding for the ORF of rat PDE7B1 was amplified from rat brain cDNA by PCR with the primer set 5'-GCGGCCGCAATGTCTTGTTTAATG-3' and 5'-TCTAGAGGACGGACAGATGGCCGT-3'. An amplified fragment was subcloned into pGEM-T Easy and sequenced, producing pG-R7B1. The *NotI*-*XbaI* fragment of pG-R7B1 was inserted into corresponding sites of the pFLAG-CMV2 expression vector, resulting in pFLAG-R7B1. The 5' region of rat PDE7B2 cDNA was amplified from rat testis cDNA by using the primer set 5'-GCGGCCGCGATGACACCGCAGG-3' and 5'-AGTCCAA-GCATGATGTCCAG-3', subcloned into pGEM-T Easy and then sequenced, producing pG-R7B2. The *NotI*-*AatII* fragment of pG-R7B2 and the *AatII*-*XbaI* fragment of pFLAG-R7B1 were ligated into the *NotI*-*XbaI* sites of pFLAG-CMV2, resulting in pFLAG-R7B2. The 5' region of rat PDE7B3 cDNA was also obtained by PCR with the primer set 5'-GCGGCCGCAATG-TCTTGTTTAATG-3' and 5'-AGTCCAAGCATGATGTCC-AG-3', with pG-R7B4N1 as a template. An amplified fragment was subcloned and sequenced, producing pG-R7B3. The *NotI*-*AatII* fragment of pG-R7B3 and the *AatII*-*XbaI* fragment of pFLAG-R7B1 were inserted into the *NotI*-*XbaI* sites of pFLAG-CMV2, resulting in pFLAG-R7B3. A putative PKA phosphorylation site located in the N-terminal regions of rat PDE7B1 and PDE7B3 was mutagenized from RRGSS to RRGAA with a QuickChange site-directed mutagenesis kit (Stratagene) and the primer set 5'-CTGCCGAACGCCGCGCGCCTA-CCCGTTTCATTG-3' and 5'-CAATGAACGGGTAGGCGCC-GCGGCGTTCGGCAG-3', resulting in pFLAG-R7B1S^{45A} and pFLAG-R7B3S^{58A} respectively. Alternatively, the C-terminal PKA phosphorylation site on each variant was mutagenized with the primer set 5'-CAGCACAGACGCAGGGGCGCCGCC-AGGACCCAG-3' and 5'-CTGGGTCCTGGCCGCGCCCC-CTGCGTCTGTGCTG-3', resulting in pFLAG-R7B1S^{426A}, pFLAG-R7B2S^{339A} and pFLAG-R7B3S^{439A} respectively. Expression plasmids encoding rat PDE7B1 and PDE7B3 mutants with Ala replacements at both N-terminal and C-terminal phosphorylation sites were constructed with the latter primer set and pFLAG-R7B1S^{45A} and pFLAG-R7B3S^{58A} as a template, resulting in pFLAG-R7B1S^{45,426A} and pFLAG-R7B3S^{58,439A} respectively. The nucleotide sequences of PDE7B mutants were confirmed by sequencing.

Expression of PDE7B in COS-7 cells

Expression plasmids pFLAG-R7B1, pFLAG-R7B2 and pFLAG-R7B3 were transfected into COS-7 cells by using LIPOFECTAMINE Plus reagent (Life Technologies) in accordance with the manufacturer's instruction. After incubation for 48 h, cells were harvested and washed three times with ice-cold PBS. The cells were disrupted by a sonicator in a homogenization buffer [20 mM Tris/HCl (pH 7.5)/2 mM magnesium acetate/0.3 mM CaCl₂/1 mM dithiothreitol/1.3 mM benzamidine/40 μ M leupeptin/0.2 mM PMSF/1 mM NaN₃] and centrifuged at 100 000 *g* for 60 min. The supernatants were collected and stored at 4 °C for further analyses.

PDE assay

The PDE assay was performed as described previously [18]. In brief, enzyme solution was incubated in PDE assay buffer [50 mM Tris/HCl (pH 8.0)/5 mM MgCl₂/4 mM 2-mercaptoethanol/0.33 mg/ml BSA] with 4 nM [³H]cAMP and various concentrations of non-radiolabelled cAMP in a final volume of 0.5 ml. The concentration of cAMP used in kinetic analysis ranged from 0.03 to 0.4 μ M. All assays were performed in triplicate; results are means $\pm$ S.D. for four independent assays with two separate protein samples.

Production of anti-PDE7B antiserum

Anti-PDE7B antiserum was raised against a synthetic peptide (WSERVCEEFYRQGELEQK) complexed with multiple antigen peptide resin (TAKO8-WTGS; Shimadzu, Kyoto, Japan). The sequence of the synthetic peptide was derived from the deduced amino acid sequence of human PDE7B. The multiple antigen peptide complex was mixed with Freund's adjuvant (Sigma) and was inoculated into Japanese White rabbits (Kitayama Laboratories, Nagano, Japan). After the rabbits had been inoculated four times over 2 months, antiserum was collected and stored at -80 °C until use.

Phosphorylation *in vitro* by PKA

COS-7 cells were transfected with FLAG-tagged expression plasmids with LIPOFECTAMINE Plus reagent. After transfection, COS-7 cells were harvested and disrupted by a sonicator in TNE buffer [10 mM Tris/HCl (pH 7.5)/1% (v/v) Nonidet P40/0.15 M NaCl/1 mM EDTA/10 μ g/ml aprotinin/10 μ M leupeptin/1 mM dithiothreitol]. High-speed supernatants (100 000 *g* for 60 min) of the transfected COS-7 cell extracts were immunoprecipitated with the anti-FLAG monoclonal antibody and Protein-G-Sepharose (Amersham Biosciences) in TNE buffer at 4 °C by rotation. After the beads had been washed with TNE buffer, phosphorylation solution [50 mM Tris/HCl (pH 7.5)/20 mM magnesium acetate/5 mM β -glycerophosphate/1 mM Na₃VO₄/200 μ M ATP/6.7 nM [³²P]ATP] and 0.8 μ g of the catalytic subunit of PKA (Promega) were added to the beads and incubated at 30 °C in the absence or presence of 10 μ M PKI 5-24 (Calbiochem), a PKA inhibitor peptide, for 30 min. The beads were then collected and resuspended in Laemmli buffer [19] and subjected to SDS/PAGE [10% (w/v) gel]. Proteins in the gels were transferred to PVDF membrane (Millipore) and exposed to X-ray film. The blots were treated with 4% (v/v) Block Ace (Snow Brand Milk Products, Tokyo, Japan) and immunoblotted with the anti-PDE7B antiserum. Detection of bands was performed with ECL[®] Western blotting detection reagents (Amersham Biosciences).

Metabolic labelling of rat PDE7B in COS-7 cells

COS-7 cells were transfected with expression plasmids encoding rat PDE7B splice variants with the use of LIPOFECTAMINE 2000 reagent (Life Technologies). After transfection for 6 h, medium was replaced with fresh labelling medium [sodium pyrophosphate-free DMEM/20 mM Hepes/0.5 mCi/ml [³²P]-orthophosphoric acid (NEN)] and incubated overnight. Labelled cells were challenged with 10 μ M forskolin for 30 min. After being washed with ice-cold PBS, cells were harvested and disrupted by sonicator in TNE buffer containing 5 mM β -glycerophosphate and 1 mM Na₃VO₄. The resulting cell extracts were immunoprecipitated with the anti-FLAG monoclonal antibody and subjected to SDS/PAGE, then analysed as described above.

RESULTS

Isolation of rat PDE7B1 cDNA

Rat PDE7B1 cDNA was amplified from rat brain cDNA with a primer set based on the nucleotide sequences of human and mouse PDE7B cDNA species. After subcloning and sequencing of the inserts, the nucleotide sequences of five independent clones were aligned to determine a consensus of the isolated cDNA. The isolated cDNA (1754 bases) contained an ORF composed of 446 amino acid residues with a molecular mass of 51.5 kDa (Figure 1). An in-frame termination codon (TAA) of rat PDE7B1 cDNA was found next to the first methionine residue. The predicted amino acid sequence of the ORF was 96.9% and 93.7% identical with those of mouse and human PDE7B respectively. Considering the N-terminal and C-terminal sequences of the isolated clone, we designated this clone rat PDE7B1. Two putative PKA

1	GTTGCTGGATTCTGCAGCACAAGTCTTCATGAACAAGCCGACCGCTCAGAGATTTCACG	60
61	GCATTCAAAGGTACAGAACTGCCACTATGGTTAAATGCTTTGTTTAAATGGTTGAGAGGT	120
121	GTGGCGAAGTCTTGTGTGAGAATCTGAGCAGAATGTCAAATGTGTTTGCATGCTAGGAG	180
181	ATGTACGACTAAGGGGTGACAGCGGGGGTCTGCGGAACGCCGCGGCTCTTACCGTTCA	240
241	TTGACTTCGCTCTACTTAACAATACAACACACTCAGGGGAAATGGCAGCAAGAAAAAGG	300
301	TGAGGAGACTGTTAAGTTTCCAAAGGCACCTTCCATGAATCTAGGCTGCTCCGGGGGATGA	360
361	CACCGCAGCGCCCCCTCCACCTGCTGAGGAGAGACTACCTTGGACAAGCAAGGCATATG	420
421	TCTCCTCAAGTTGGAATGTGGGACTTTGACATTTTCTTGTGTTGATGCTTGCAGAAATGGGA	480
481	ACAGTCTGGTAACCTGTTGTGTCACCTCTTCAACTCCGATGGACTATCCACCAATTTCA	540
541	AGCTTGACATGGTACCTTACACAGGTTTTTGGTTATGTTTACAGGAAGATTACACGGCC	600
601	ACAACCCGTACCACAATGCTGTTTCATGACGTGAGCTCACCAGGCCATGCACTGTTACT	660
661	TGAAGGAGCCAAAGTTGGCGAGCTTCTCTACACCTCTGGACATCATGCTTGGACTATTGG	720
721	CTGCAGCAGCTCATGACGTGGACCAACCCAGGGGTGAACCGCATTTTGTATCAAACTA	780
781	ACCACCACTTGGCAACTTGTATCAGAACATGTCGCTACTGGAGAATCACCAGCTGGCGGT	840
841	CTACAATCGGCACTGCTTCGGGAATCAAGGCTCCTTGTCTATTGCGCAAGGAAATGACAC	900
901	AGGATATCGAAGCAGCAGCTGGGCTCCCTGATCTTGGCCACGGACATCAACAGACAGAATG	960
961	AGTTCTCTGACCCGCTTAAAGCTCACCTCCCAATAAGGATTTGAGACTGGGAAATATAC	1020
1021	AGGACAGCAGCTTTATGCTTCAGCTCGCTTGAAGTGCGCTGACATTTGCAATCCTTGTG	1080
1081	GAATCTGGGAGATGAGCAAGCAGTGGAGTGAAGAGTCTGCGAAGAAATCTCAGGCGAAG	1140
1141	GTGACCTTGAACAGAGTTTGAACCTGGAATCAGTCCCTCTTGTATCAACAGAGAAATG	1200
1201	CAATCCCTAGCATACAATTTGGTTTCATGACTTACATCGTGGAGCCACTGTTCGGGAGT	1260
1261	GGGCCCGGTTCACTGGGAACAGCAGCCCTGTCGAGAGCATGCTAAACCATCTCGCGCAC	1320
1321	ACAAGCCAGCTGGAAGAGCTGCTGTCATTCAGTACAGACAGCAGGGGACGCGCCAGG	1380
1381	ACCACAGCGGGCAGCGCGCTGAGACCTTGAGCAGACAGAGGCCACGCGCTTAAGGTA	1440
1441	D P A G T A P E T L E Q T E G A T P *	1500
1501	CCCTCGCAACAAGCCACCGCGCTGGGTTTCAAGATGCCACCTGCCTGCCATTATACCACC	1560
1561	TCCCTTCGTTGATCCAAGTGACAAAAGCCATCGTCACTCAGCATTAGCTGCCGAAATG	1620
1621	GGCAGCTCTATCTGTCTATTGTSAGCTGATTAATCTGGGGCGTCTGCCCCACCGAAAAG	1680
1681	ACTGGAAGTAAGAAAGGGTGCTCTGCGTGCCCGCTCTGGCCCTTGGTCACGCTGAC	1740
1741	TGGCAGTAGCTCCT	1754

Figure 1 Nucleotide and predicted amino acid sequences of rat PDE7B1 cDNA

The deduced amino acid sequence of rat PDE7B1 is shown in single-letter code below the nucleotide sequence. The boxed sequence indicates the putative catalytic domain of PDE7B1. The termination codon at the end of the ORF is represented by an asterisk. Putative phosphorylation sites for PKA are underlined. The in-frame termination codon located upstream of the first methionine is doubly underlined.

Figure 2 Northern blot hybridization of rat PDE7B transcripts in various tissues

All blots were hybridized with a 32 P-labelled probe of rat PDE7B cDNA under the conditions described in the Materials and methods section. (A) Northern blot analysis of rat MTN blot (ClonTech). The sizes and positions of RNA size markers are shown at the left. (B) Northern blot analysis of poly(A)⁺ RNA species from various regions of 10 week-old rat brain. Poly(A)⁺ RNA species (2 µg per lane) were separated in a 1% (w/v) agarose/0.66 M formamide gel, transferred to Hybond N⁺ membrane and then hybridized with the probe.

phosphorylation sites, RRGs⁴⁵ and RRGs⁴²⁶, were found in the N-terminal and C-terminal regions of rat PDE7B1 respectively.

Northern hybridization analysis of rat PDE7B transcripts

Northern hybridization analysis was performed to examine the tissue distribution of rat PDE7B transcripts by using a rat PDE7B1 cDNA (nt 67–443) as a probe. Strong hybridization signals were detected in the testis; the next strongest signals were in the brain (Figure 2A). Moderate signals were observed in the heart, lung, skeletal muscle and kidney. The size of rat PDE7B transcripts in these tissues was approx. 5.5 kb. However, major transcripts in the testis (approx. 2.0 kb) were apparently shorter than those in other tissues. To investigate the detailed expression pattern of rat PDE7B transcripts in the brain, Northern hybridization analysis was performed with the same probe as that used above (Figure 2B). Rat PDE7B transcripts were particularly abundant in the striatum. Moderate expression was observed in the cerebral cortex and midbrain. Weak signals were found in the cerebellum, brainstem, olfactory bulb and hippocampus.

Novel N-terminal splice variants of rat PDE7B in the testis and skeletal muscle

The length of rat PDE7B transcripts in the testis was shorter than that in the other tissues, suggesting the presence of splice variants of rat PDE7B. To obtain cDNA species coding for novel splice variants, we performed 5' RACE with a Marathon-Ready cDNA library (rat testis) as a template. An amplified RACE product was subcloned and then sequenced. The resultant six independent clones were revealed to possess a novel sequence coding for a unique N-terminal variant of rat PDE7B by comparison with the

Figure 3 Comparison of nucleotide and predicted amino acid sequences of rat PDE7B variants

(A) Alignment of nucleotide and amino acid sequences of N-terminal region of rat PDE7B splice variants. Amino acid sequences of rat PDE7B1, PDE7B2 and PDE7B3 are shown above the respective nucleotide sequences. The common sequence for rat PDE7B variants is represented by shaded boxes. An in-frame termination codon (TGA) of rat PDE7B2 cDNA upstream of the first methionine is underlined. The 5' non-coding sequence of rat PDE7B2 is represented by lower-case letters. (B) A diagram of rat PDE7B splice variants. Arrows indicate PKA phosphorylation sites located at the N-terminus and C-terminus of rat PDE7B variants. Abbreviation: a.a., amino acids.

rat PDE7B1 sequence. A full-length cDNA for the novel splice variant was obtained by PCR. The sequences of four independent clones were aligned to determine a consensus. A sequence analysis of the ORF for the novel rat PDE7B variant predicted 359 residues with a molecular mass of 41.5 kDa. The isolated cDNA contained a 5' region distinct from rat PDE7B1 cDNA. We therefore designated the cDNA isolated from testis as rat PDE7B2. An in-frame termination codon (TAA) was found 207 bp upstream of the first methionine residue (Figure 3A). It is noteworthy that rat PDE7B2 was devoid of an N-terminal PKA phosphorylation site (Figure 3B), which is present in rat PDE7B1 and in mouse and human PDE7B.

For the identification of a full-length ORF of rat PDE7B1 in various tissues, we performed PCR with a rat cDNA library of MTC panel I. During the course of this study, one clone distinct from rat PDE7B1 was obtained from rat skeletal muscle cDNA with a primer set for rat PDE7B1. A sequence analysis showed that the clone encoded a novel sequence of rat PDE7B, which contained an additional 39 nt (13 residues) within the N-terminal region of rat PDE7B1 (Figure 3A). The ORF of this variant predicted 459 residues with a molecular mass of 52.7 kDa. We defined this variant as rat PDE7B3.

Tissue-specific expression of rat PDE7B variants

The expression of rat PDE7B variants in various tissues was investigated by PCR with specific primers for each variant and rat MTC panel I as templates (Figure 4). A band derived from PDE7B1 transcripts was detected in the heart, brain, spleen, lung, skeletal muscle and kidney. PDE7B3 transcripts were

Figure 4 Expression of three types of rat PDE7B splice variant in various tissues

PCR was performed with a rat MTC panel as templates under the conditions described in the Materials and methods section. Amplification was conducted for 33 cycles (upper panel) and 30 cycles (lower panel). PCR products, which were obtained with control plasmids as templates under the same conditions, are shown on the right. The concentrations of control plasmids used were 1 pg/ml for pFLAG-R7B1 and pFLAG-R7B3 (upper panels) and 10 pg/ml for pFLAG-R7B2 (lower panels).

expressed in the heart, lung and skeletal muscle. In contrast, PCR with a primer set for PDE7B2 transcripts demonstrated that the expression of PDE7B2 transcripts was confined to the testis. To estimate the relative amounts of these three variants, the efficiency of PCR amplification of each primer set used was examined by using the expression plasmids pFLAG-R7B1, pFLAG-R7B2 and pFLAG-R7B3 as templates under the same

Figure 5 *In situ* hybridization analysis of PDE7B in rat testis

Localization of rat PDE7B transcripts in rat testis was revealed by *in situ* hybridization with digoxigenin-labelled anti-sense and sense probes. (A) Positive staining of seminiferous tubules with anti-sense probe. (B) Sense probe hybridization of corresponding region of (A). (C) Hybridization signal of the seminiferous tubule at stage XIV. (D) Haematoxylin–eosin staining of the seminiferous tubules at stage XIV. Scale bars, 120 μm (A, B) and 30 μm (C, D).

PCR conditions. Considering the cycle number and the amounts of templates, the primer set for rat PDE7B2 transcripts was approx. 6-fold less efficient than that for PDE7B1 and PDE7B3 transcripts (Figure 4). We therefore estimate that the relative amount of PDE7B2 transcripts in the testis was greater than those of PDE7B1 and PDE7B3 transcripts. The amounts of PDE7B3 transcripts were lower than those of PDE7B1 transcripts in the heart, lung and skeletal muscle.

***In situ* hybridization of PDE7B transcripts in testis and brain**

In situ hybridization was performed to examine the cellular localization of PDE7B transcripts in rat testis and brain. In the testis, seminiferous tubules at stages XII–XIV were stained with the anti-sense probe (Figures 5A and 5C). Furthermore, expression of PDE7B transcripts in the testis was observed in pachytene spermatocytes during meiotic prophase and in meiotic germ cells. No specific staining was detected with the sense probe (Figure 5B). Figure 5(D), which is a serial section of Figure 5(C), shows haematoxylin–eosin staining of the seminiferous tubule at stage XIV. In the brain, strong hybridization signals with the anti-sense probe were observed in the striatum (Figures 6A, 6C and 6D), in accord with the result of Northern hybridization. Moderate signals were also observed in the olfactory nucleus (dorsal) and posterior thalamic nucleus (Figures 6E and 6G). By contrast, no hybridization signal was detected in these regions of rat brain with the sense probe (Figures 6B, 6F and 6H).

Kinetic properties and inhibitor sensitivity of recombinant rat PDE7B

K_m values of FLAG-tagged rat PDE7B splice variants expressed in transfected COS-7 cells were determined from Lineweaver–Burk plots. The K_m values of recombinant rat PDE7B1, PDE7B2 and PDE7B3 for cAMP were 0.05 ± 0.004 , 0.07 ± 0.01 and 0.05 ± 0.01 μM respectively. The inhibitory effects of several PDE inhibitors on recombinant rat PDE7B variants were examined (Table 1). Recombinant rat PDE7B1 was the most sensitive to IBMX, with an IC_{50} value of 1.5 μM , followed by SCH51866 (IC_{50} 2.5 μM) and dipyridamole (IC_{50} 5.0 μM). IBMX also inhibited rat PDE7B2 and PDE7B3 with IC_{50} values of 2.1 and 1.6 μM respectively. SCH51866 inhibited recombinant rat PDE7B2 and PDE7B3 with IC_{50} values of 2.1 and 2.9 μM respectively. Dipyridamole inhibited recombinant rat PDE7B2 and rat PDE7B3 with IC_{50} values of 5.0 and 6.1 μM respectively. All recombinant rat PDE7B splice variants were insensitive to erythro-9-(2-hydroxy-3-nonyl)-adenine, milrinone and rolipram at 100 μM .

Phosphorylation of rat PDE7B by PKA

Anti-PDE7B antiserum was raised against a synthetic peptide derived from the deduced amino acid sequence of a common region of PDE7B variants. To examine the specificity of the anti-PDE7B antiserum, FLAG-tagged recombinant rat PDE7B1

Figure 6 *In situ* hybridization of PDE7B in rat brain

In situ hybridization of rat brain was performed with digoxigenin-labelled anti-sense (A, C–E, G) and sense (B, F, H) probes of rat PDE7B. (A) Positive staining of the striatal neurons with an anti-sense probe. (B) Sense probe hybridization of corresponding region of (A). (C) A magnified image of positively stained striatal neurons shown in (A). (D) Positive and negative signals of striatum (st) and globus pallidus (gp) respectively. (E) Hybridization signal in the olfactory nucleus (dorsal) with an anti-sense probe. Positive staining is indicated by arrowheads. (F) Sense probe hybridization of serial section of (E). (G) Positive staining of the posterior thalamic nucleus. (H) Sense probe hybridization of serial section of (G). Scale bars, 300 μ m (A, B, D, E–H) and 60 μ m (C).

expressed in COS-7 cells was analysed by immunoblotting with the anti-FLAG monoclonal antibody or the anti-PDE7B antiserum (Figure 7A). Protein of molecular mass approx. 52 kDa was detected with both anti-FLAG monoclonal antibody and anti-PDE7B antiserum, which is in reasonable agreement with the prediction from the calculated molecular mass of the FLAG-tagged rat PDE7B1.

PKA-dependent phosphorylation of rat PDE7B splice variants was examined by a phosphorylation assay *in vitro* with FLAG-tagged recombinant proteins. As shown in Figure 7(B), each rat PDE7B splice variant was phosphorylated by PKA. The PKA-dependent phosphorylation was completely inhibited in the presence of PKI 5–24. To investigate the PKA phosphorylation sites of rat PDE7B variants, FLAG-tagged rat PDE7B variants

Table 1 Inhibition studies of recombinant rat PDE7B variants with various PDE inhibitors

Recombinant proteins expressed in COS-7 cells were used to determine the inhibitory effects of various PDE inhibitors. IC₅₀ values were calculated by linear regression. All assays were performed in triplicate at a cAMP concentration of 0.1 μM. Results are means ± S.D. for four independent assays with two separate protein samples. Abbreviation: EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine.

Inhibitor	IC ₅₀ (μM)		
	Rat PDE7B1	Rat PDE7B2	Rat PDE7B3
IBMX	1.5 ± 0.26	2.1 ± 0.45	1.6 ± 0.57
EHNA	> 100	> 100	> 100
Milrinone	> 100	> 100	> 100
Rolipram	> 100	> 100	> 100
Dipyridamole	5.0 ± 0.45	5.0 ± 0.78	6.1 ± 0.13
SCH51866	2.5 ± 0.28	2.1 ± 0.56	2.9 ± 0.16

containing an alanine replacement at their C-terminal PKA phosphorylation site (RRGS to RRGA) were generated and subjected to a phosphorylation assay *in vitro*. PKA-dependent phosphorylation of rat PDE7B2 was abolished by the introduction of the alanine replacement at the C-terminal PKA phosphorylation site (Figure 7C), indicating the phosphorylation of PDE7B2 at Ser³³⁹. In contrast, the mutants with a single alanine replacement at the C-terminal [rat PDE7B1(S426A) and PDE7B3(S439A)] or N-terminal [PDE7B1 (S45A) and PDE7B3(S58A)] PKA site were still phosphorylated by PKA (Figures 7C and 7D). Double mutants lacking both N-terminal and C-terminal phosphorylation sites were not phosphorylated (Figure 7E). These observations indicate that both N-terminal and C-terminal phosphorylation sites of rat PDE7B1 and PDE7B3 were active in PKA phosphorylation *in vitro*.

To investigate whether rat PDE7B splice variants could be phosphorylated *in vivo*, metabolic labelling of these variants was performed with COS-7 cells transfected with expression plasmids

Figure 7 Phosphorylation of rat PDE7B variants by PKA

(A) Detection of FLAG-tagged recombinant rat PDE7B1 by immunoblot analysis with the anti-PDE7B antiserum or the anti-FLAG monoclonal antibody. The positions of protein molecular mass markers are shown at the left. (B) PKA-dependent phosphorylation of wild-type (wt) recombinant rat PDE7B splice variants. (C, D) Phosphorylation assay *in vitro* of mutants of rat PDE7B splice variants carrying an alanine substitution at the C-terminal (C) or the N-terminal (D) PKA phosphorylation site. (E) Phosphorylation of two mutants having alanine replacements at both phosphorylation sites. Extracts from transfected COS-7 cells were immunoprecipitated with the anti-FLAG monoclonal antibody and the immunoprecipitates were used in a phosphorylation assay *in vitro*. After all membranes had been exposed to X-ray film (³²P), they were analysed by immunoblotting with the anti-PDE7B antiserum. Abbreviations: IB, immunoblotting; PKI, PKI 5–24.

Figure 8 Phosphorylation of rat PDE7B splice variants *in vivo* in COS-7 cells

[^{32}P] P_i was incorporated into transfected COS-7 cells; the cells were then treated for 30 min with 10 μM forskolin. Recombinant proteins were immunoprecipitated with the anti-FLAG monoclonal antibody. A membrane was exposed to X-ray film (^{32}P), then analysed by immunoblotting (IB) with the anti-PDE7B antiserum.

encoding rat PDE7B variants. As shown in Figure 8, all three recombinant proteins of rat PDE7B splice variants were labelled *in vivo*. These results indicate that rat PDE7B splice variants are potentially phosphorylated under physiological conditions.

DISCUSSION

In the present study we isolated rat PDE7B1 and two novel splice variants, rat PDE7B2 and PDE7B3, that exhibited unique tissue-specific expression and phosphorylation. Rat PDE7B1, composed of 446 residues, was highly similar to mouse PDE7B1 in the amino acid sequence [17]; rat PDE7B2 was a testis-specific PDE7B variant of 359 residues. Rat PDE7B3, 459 residues in size, contained an insertion of 13 residues in the N-terminal region of rat PDE7B1. Three different variants exhibited similar K_m values for cAMP. These values agreed well with those of the human (K_m 0.13 μM) and mouse PDE7B (K_m 0.03 μM) enzymes reported previously [16,17].

Rat PDE7B transcripts were particularly abundant in the testis and striatum. It has been reported that human PDE7B transcripts are abundant in the caudate nucleus [16] and that mouse PDE7B transcripts are highly expressed in the pancreas [17]. With regard to the testis, PDE7B transcripts have not been detected in either humans or mice. The PDE7B gene is likely to be regulated by species-specific mechanisms. Rat PDE7B transcripts in the testis were approx. 2.0 kb in length, whereas those in other tissues were larger (approx. 5.5 kb). Rat PDE7B1 and PDE7B3 transcripts were not detected in the testis, and PDE7B2 transcripts were restricted to the testis, indicating that highly abundant short transcripts of approx. 2.0 kb in the testis are PDE7B2. As shown in Figure 3(A), variation in the N-terminal region in rat PDE7B2 and PDE7B3 is observed at a specific site, nt 178 in rat PDE7B1, suggesting that RNA splicing occurred at this position.

Two putative phosphorylation sites for PKA are identified at the N-terminus and C-terminus of rat PDE7B1 and PDE7B3, whereas rat PDE7B2 lacks the N-terminal site. The three PDE7B splice variants are a good substrate for PKA *in vitro*. Furthermore, these splice variants can be phosphorylated *in vivo* by PKA. PKA-dependent phosphorylation and subsequent alteration of enzymic properties of PDEs [20–23] and their translocation [24] have been elucidated. Splice-variant-specific regulation of enzymic activity of PDE4D by phosphorylation has been well studied. PDE4D1 is stimulated by ERK2 phosphorylation

at the C-terminus of a catalytic domain, whereas PDE4D2 and PDE4D3 are inhibited by the phosphorylation [25]. However, PKA-dependent phosphorylation at the upstream conserved region 1, located within a splice region of PDE4D3, results in the ablation of the ERK2-induced inhibition [15,25]. It is likely that the presence of an N-terminal phosphorylation site for PKA in rat PDE7B1 and PDE7B3 implies a distinct alteration of enzymic properties of three PDE7B variants.

cAMP-dependent signal transduction systems are important in spermatogenesis [26]. It is intriguing that transcripts of PDE7B2, a cAMP-specific PDE, were expressed in the testis. *In situ* hybridization demonstrated that hybridization signals of rat PDE7B transcripts were confined to the germ cells of the seminiferous tubules at stages XII–XIV. It has been reported that an increase in cAMP concentration in the seminiferous tubules begins at stage XII [27,28]. These observations suggest that the expression of PDE7B2 might be coincident with the stage at which cAMP elevation is evoked and might contribute to controlling the cAMP concentration during subsequent stages. In the previous studies, stage-specific expression of PDE8A transcripts [29] and PDE4 [30,31] during spermatogenesis have also been reported. Rolipram-insensitive PDEs have been reported to increase during stages IX–XII in rats [30], suggesting the presence of PDE7B2 in these stages. Our results showed that rat PDE7B2 is different from rat PDE7B1 and PDE7B3 in PKA-dependent phosphorylation. The stage-specific expression and splice-variant-specific phosphorylation of rat PDE7B2 suggest an involvement of rat PDE7B2 in spermatogenesis via cAMP metabolism.

In the brain, PDE7B1 transcripts are highly expressed in the striatum. In striatal neurons, the concentration of intracellular cAMP is regulated by dopamine via dopamine D1 and D2 receptors. D1 receptor is coupled to adenylate cyclase and stimulates the subsequent cAMP/PKA pathway. Previous reports have shown that a dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) enriched in striatal neurons [32] is phosphorylated by PKA and then activated. Phospho-DARPP-32 inhibits protein phosphatase 1 ('PP1') [33], resulting in the potentiation of L-type Ca^{2+} channels [34] and N-methyl-D-aspartate receptor [35]. In Huntington transgenic mice, a decrease in D1 receptor [36] and the attenuation of the subsequent dopamine-signalling cascade [37] are observed in striatal neurons. Positron-emission tomography ('PET') studies of the striatum showed a decrease in striatal D1 and D2 receptors in Huntington's disease patients [38]. These observations indicate that the dopamine/cAMP signalling pathway is essential to neural activities in the striatum. PDE1B, PDE4 and PDE10A, in addition to PDE7B transcripts, are also expressed in the striatum [18,39–41]. Of the PDEs present in the striatum, PDE7B1 shows the lowest K_m value for cAMP. The presence of several cAMP-hydrolysing PDEs with different K_m values implies the critical regulation of various neural functions in the striatum according to the cAMP levels through hydrolysis. It has been reported that D1 and D2 receptors are expressed in different spiny neurons in the striatal medium [42,43]. It is therefore intriguing to consider which dopamine receptor co-localizes with which PDE(s). Detailed studies such as double staining will lead to a further understanding of cyclic nucleotide metabolism in striatal neurons.

Although the presence of enzymic activity of PDE7B *in vivo* remains unclear, tissue-specific expression and PKA-dependent phosphorylation of PDE7B splice variants suggest that these variants have specific roles in controlling cellular functions via cAMP signalling in these tissues. The discovery of specific inhibitors for PDE7B might explain the physiological functions of this enzyme in tissues.

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