Molecular cloning and functional expression in yeast of a human cAMP-specific phosphodiesterase subtype (PDE IV-C)

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Abstract We have recently reported increased survival of dopaminergic substantia nigra neurons by inhibition of phosphodiesterase type IV enzymes. As a first step to unravel the involvement of PDE IV subtypes in this process, we isolated phosphodiesterase type IV cDNAs from human substantia nigra. One isolated partial cDNA clone was most homologous to the partially cloned rat and human PDE IV-C isogene. Distribution analysis revealed that the enzyme is expressed in various tissues but not in cells of the immune system. Isolation of the full-length human PDE IV-C isogene cDNA and expression in a PDE-deficient yeast strain resulted in functional complementation of the yeast heat shock response. Inhibition of the enzymatic activity by rolipram characterized this enzyme as a typical type IV phosphodiesterase.

Key words: Substantia nigra; Rolipram; Yeast complementation; SH-SY5Y cell

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) regulate the cellular concentration of cyclic nucleotides by converting cAMP or cGMP to the non-cyclic 5′-NMP [1]. The PDE enzyme family can be divided into 7 subfamilies or classes [2,3], characterized by their substrate affinity and specificity and their sensitivity to specific inhibitors [1,4,5] and activators. The subfamilies are Ca^{2+}/Calmodulin-dependent PDEs (PDE I), cGMP stimulated PDEs (PDE II), cGMP inhibited PDEs (PDE III), cAMP-specific rolipram sensitive PDEs (PDE IV), cGMP specific PDEs (PDE V), photoreceptor PDEs (PDE VI), and high affinity cAMP-specific rolipram-insensitive PDEs (PDE VII). For all the families, multiple related genes as well as different mRNA splice forms create a basis for variable control of cyclic nucleotide concentration in different cells and subcellular compartments [6,7].

One interesting family with respect to therapeutic applications is PDE IV. Inhibitors of this enzyme class may be beneficial in asthma [8] and other inflammatory diseases [9] as well as in diseases of the CNS like depression [10] or multiinfarct dementia [11]. Recently, we demonstrated that cyclic AMP stimulated the dopamine uptake and development of mesencephalic dopaminergic neurons in primary cell cultures [12]. In addition, an elevated intracellular level of cAMP, but not bFGF or IGF-I, prevented the cultured neurons from degeneration and protected them from the dopaminergic neurotoxin MPP^+ [12]. In a previous study with inhibitors for different phosphodiesterase subtypes we found, that PDE type IV specific inhibitors also increased the survival of dopaminergic neurons in primary cell culture and protected mice from the action of the neurotoxin MPTP [13,14].

In order to characterize the PDE IV subtypes involved in that process we isolated PDE IV cDNAs from a human substantia nigra cDNA library. One of the subtypes we found in this tissue had previously only been described as a short partial DNA fragment. We have now cloned and characterized the full-length cDNA. After expression in yeast the recombinant protein displayed cAMP-specific activity and was sensitive to rolipram. The spatial expression of this gene was distinct from other known human PDE IV genes [15,16,17,18].

2. Materials and methods

2.1. Oligonucleotides

PE 1: 5′-GTCCAAACACATGACCCTCCTGGCTGACCTG-3′; *PE 2:* 5′-GCAGGAGGGAGCTGATTGCTGGATGAAG-3′; *PE 3:* 5′-TCAGAGCTGGCGCTTATGTAC-3′; *PE 4:* 5′-CCGTATGCTTGTCACACAT-3′; *PE 5:* 5′-TCAAGCTGCTGCAGCAGAG-3′; *PE 6:* 5′-GAGTCCTTCCTGTACCGCTCA-3′; *PE 7:* 5′-GACTGGAGCCTGCATAATCCG-3′; *PE 8:* 5′-AGGTCAAAGCGCCTGCAGGAGG-3′; *PE 9:* 5′-GCCAGTCTGCGGACCGTT-3′; *PE 10:* 5′-CAGCAATGCCCTAGGAGCAGC-3′.

2.2. Cloning of PDE IV cDNA

To isolate PDE IV isogenes expressed in the substantia nigra, we designed the 30 bp oligonucleotide PE 1 which was complementary to the highly conserved catalytic domain of all published rat and human PDE IV isogenes. With this oligonucleotide probe we screened three million independent clones of a commercial λ-Zap human substantia nigra cDNA library (Stratagene) using standard procedures. DNA sequencing was performed with the T7 sequencing kit (Pharmacia). Using RT-PCR as described below, we found that SH-SY5Y neuroblastoma cells contain mRNA of the particular PDE isogene detected by PE1. To isolate a full-length cDNA clone we extracted mRNA from SH-SY5Y cells. cDNA was prepared from 3 μg poly(A)$^+$ mRNA using the Amersham cDNA kit. The size selected cDNA (> 1.5 kb) was cloned into a modified pBluescript vector as described by Foguet et al. [19]. 25 pools, each containing 4×10^5 independent clones with an average insert size of 2 kb, were obtained after transformation into the *E. coli* strain MC1061 by electroporation (Gene Pulser, Bio-Rad). From each pool, 0.5 μg DNA was used for a PCR analysis which specifically detected the cDNA clone isolated from the sustantia nigra library. Three positive pools were then plated and the clones isolated by standard filter hybridization [20] using oligonucleotide PE 1 as probe.

To extend the 5′ end, double-stranded SH-SY5Y cDNA was synthesized with the primer PE 4. After circularization of the cDNA with T4-DNA ligase, sequential inverse PCRs [21,22] were performed with the nested primer pairs PE 2/PE 3 and PE 2/PE 5. In an additional extension step, inverse PCR was performed using cDNA created with the primer PE 2. Nested primer pairs in this process were PE 6/PE 7, PE 8/PE 9, and PE 8/PE 10. The DNA fragments obtained were cloned

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into pUC18 and sequenced with the T7 sequencing kit from Pharmacia. The DNASIS and PROSIS programs (Hitachi) were used for sequence analysis.

2.3. Distribution analysis

A Northern blot (Clontech) with 2 μg poly(A)$^+$ RNA from different human tissues was hybridized with a PDE IV-C specific riboprobe (derived from nucleotide 573 to nucleotide 888 of the cDNA) in: 1 mM EDTA, 0.5 M $NaHPO_4$ pH 7.2, 7% SDS, 1% BSA, 50% formamide at 42°C. The blot was washed under stringent conditions with 0.1 × SSC, 0.1% SDS at 70°C and 80°C for 30 min each.

For RT-PCR, total mRNA was extracted, extensively treated with DNAse I and reverse transcribed into cDNA as described [23]. All RNAs were tested for genomic contamination by RT-PCRs in which the reverse transcriptase was omitted (data not shown). To determine the expression pattern of the cloned enzyme, two oligonucleotides, PE 3 and PE 4, were used which detect specifically transcripts for hPDE IV-C and hPDE IV-A, but not for PDE IV-B or PDE IV-D. PCR reactions were performed in 50 μl in the presence of 1 μCi [α-^{32}P]dCTP and 10 pmol each of the primers PE 3 and PE 4. Taq-DNA polymerase was purchased from Boehringer-Mannheim. After restriction with *Eco*RV which cuts specifically in the hPDE IV-C cDNA, or *Bst*XI which digests only PCR fragments derived from hPDE IV-A cDNA, the DNA fragments were separated on 4% agarose gels. The gels were dried and exposed to X-ray films (Kodak).

2.4. PDE expression assay

The cDNA was cloned into the extrachromosomal pYEMS2 yeast expression vector (M. Sullivan, unpublished). pYEMS2 contains the *ura3* gene as selectable marker and a cassette containing the constitutively expressing glyceraldehyde-3-phosphate dehydrogenase (GPD-) promotor and 3-phosphoglycerate kinase (PGK-) terminator spaced by a multiple cloning site. The hPDE IV-C cDNA was ligated into the *Bam*HI/*Sal*I restriction sites behind the GPD promotor. This construct, pYPDE-IVC, was transformed into the PDE deficient yeast strain YMS5. Both PDE genes in this yeast strain had previously been interrupted by inserting the selection markers LYS2 and LEU2 into the coding sequences of the yeast PDE 1 and PDE 2 genes, similar to the strains described by McHale et al. [21]. Although PDE deficient yeast strains grow normally under standard conditions, they are more sensitive to heat shock (3 min, 55°C). Yeast transformants expressing an exogenous functional PDE IV gene reveal normal growth and heat shock resistance [3,24].

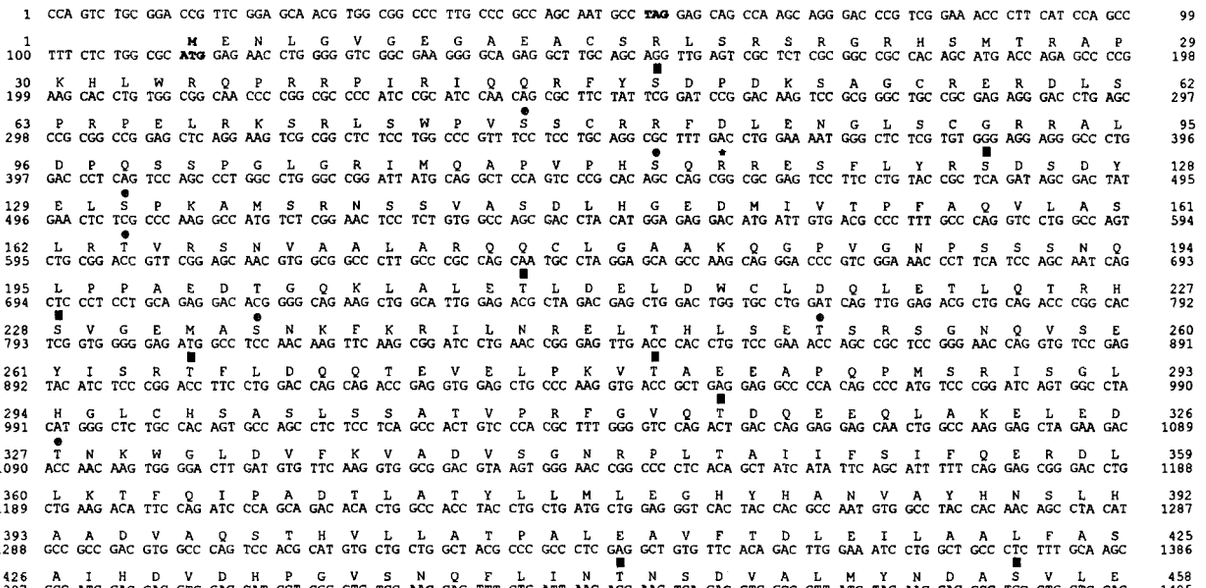

Fig. 1. Nucleotide and deduced amino acid sequence of hPDE IV-C. Amino acids below various symbols correspond to motifs for phosphorylation (⊕ = cAMP-dependent protein kinase; ★ = protein kinase C; ■ = casein II protein kinase) which may be important in the regulation of enzyme activity. The accession number of the EMBL data library is: Z46632.

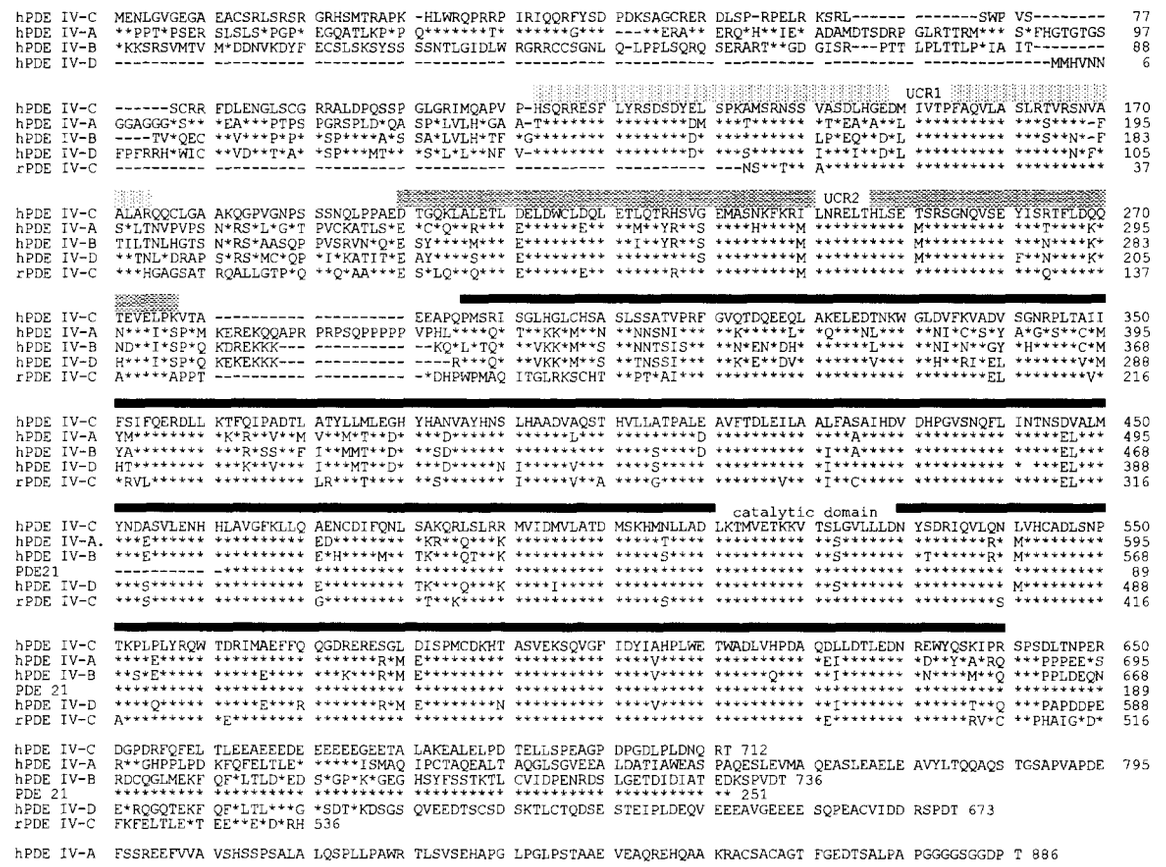

Fig. 2. Alignment of the deduced human PDE IV-C amino acid sequence with the three other full-length human PDE IV amino acid sequences (PDE 46, TM72, PDE43 [17]) and the previously described partial rat rPDE IV-C [26] and human (PDE21[17]) PDEIV-C sequences. The conserved catalytic and N-terminal UCR 1 and UCR 2 motifs are indicated.

2.5. Enzyme isolation and pharmacological analysis

Pelleted yeast (5 ml) was suspended in 50 ml of buffer (10 mM tris-hydroxymethyl-aminomethane, 1 mM ethylenediamine-tetraacetic acid, 1 μg/ml each of leupeptin and pepstatin A, 175 μg/ml phenylmethyl-sulphonyl fluoride, 1 mM dithiothreitol, pH 7.4 with HCl). After centrifugation, 15 g of glass beads (425–600 μm, acid-washed; Sigma) washed with buffer were added to the pellet. The slurry containing the glass beads was vigorously agitated for 4 h at 4°C after the addition of 1 ml of buffer and 60 mg of cholamidopropane sulphonic acid were added to the slurry. Disintegration of the yeast cells, usually >50%, was observed by phase-contrast microscopy. The slurry was subsequently transferred to a coarse glass funnel, collected by suction and washed with a total of 15 ml buffer. The flow-through was separated by centrifugation (2000 × *g*, 10 min, 4°C). The pellet was resuspended in 15 ml of buffer. PDE activity was determined in this suspension and in the cytosolic supernatant.

The assay protocol was based on the two-step method described by Thompson et al. [25] modified for 96-well microtitre plates. Briefly, enzyme was diluted with homogenization buffer (see above) such that 10–30% total substrate hydrolysis was obtained during the assay. To start the reaction, 25 μl of diluted enzyme was added to 100 μl of substrate ([^{3}H]cAMP, 1.25 μM, 740 Bq). After 30 min at 37°C, the reaction was stopped in a hot water bath (65°C, 5 min). Plates were cooled on ice and incubated for 10 min at 37°C with 25 μl of nucleotidase (0.1 mg/ml in water; snake venom from *Oiophaghus hannah*). The unreacted substrate was separated from [^{3}H]adenosine by sequentially adding aliquots (100 + 50 + 50 μl, at 5 min intervals) of 30% (v/v) Dowex 1 × 2 slurry (acetate form) in 0.2% (v/v) acetic acid. The Dowex and the bound cAMP was pelleted by centrifugation (150 × *g*, 5 min). Aliquots of the supernatants were transferred onto 96-well, solid-phase scintillation plates (LumaPlate, Canberra Packard) using an automated pipetting device (Hamilton MicroLab 2200), dried for >4 h at 60°C, and counted (Canberra Packard TopCount). Concentration–inhibition curves were established using graded inhibitor concentrations in presence of constant solvent concentrations (50 μl DMSO/ml final assay mixture). IC_{50} values were estimated using non-linear least squares fitting to the two-parameter logistic equation (MicroCal ORIGIN).

3. Results

3.1. Cloning of a PDE type IV isogene from human substantia nigra

Screening of three million independent clones of a commercial λ-Zap cDNA library from human substantia nigra with a PDE IV-specific oligonucleotide revealed one clone with an insert size of 450 bp. Sequence analysis showed highest homology to the partially cloned rat PDE IV-C isogene. RT-PCR analysis of various cell lines displayed that this human isogene is expressed in neuroblastoma SH-SY5Y cells. To obtain the 5′ and 3′ ends of the isolated gene, we screened a cDNA library derived from SH-SY5Y cells. Although we isolated a 3 kb cDNA clone, no in-frame stop codon was present at the 5′ end. The complete open reading frame was then obtained after two sequential inverse PCR steps with SH-SY5Y cDNA. The fragments of the 5′-end obtained by this procedure were combined with the 3 kb clone, finally resulting in a 3495 bp cDNA clone with an in-frame 5′ stop signal (Fig. 1). The sequence contained a complete open reading frame of 2136 bp corresponding to a protein of 712 amino acids (Fig. 1). Nucleotide comparisons

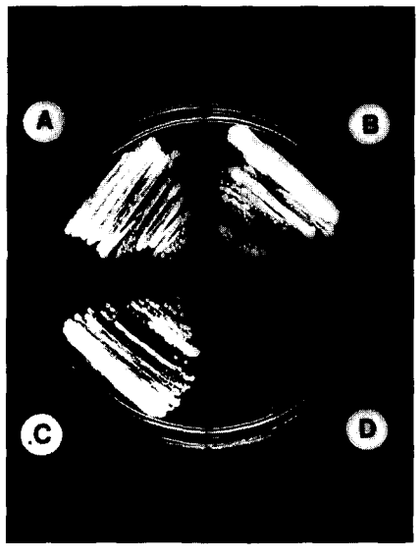

Fig. 3. Heat shock assay for functional hPDE IV-C expression in yeast. A/C = PDE deficient yeast containing pYEMS IV-C without/with heat shock; B/D = PDE deficient yeast without/with heat shock. Cells were grown after the heat shock (3 min, 55°C) for 16 h at 30°C in 1 ml of liquid medium. 2 μl were then plated on agar medium and incubated for 18 h at 30°C.

with the rat and human PDE IV genes showed highest homology between this cDNA and the rat PDE IV-C partial clone of 1736 bp [26]. While this work was in progress, a human cDNA fragment of 1155 bp identical with the 3′ part of the catalytic region of this PDE-type was described [17]. The amino acid sequence deduced from our clone displayed 82.9% overall sequence identity to the rat PDE IV-C [26,27], 73.2% to hPDE IV-A, 67.5% to hPDE IV-B and 72.8% to hPDE IV-D [17], respectively. Therefore we suggest that the clone represents the human homologue of the rat PDE IV-C. The human IV-C cDNA contains two conserved regions, UCR1 and UCR2, also found upstream of the catalytic domain in all other cloned human PDE IV isogenes. The sequence contains two consensus sequences for cAMP-dependent protein kinase, one each in the UCR1 region and the catalytic domain and several consensus motifs for protein kinase C and casein kinase II. An alignment of the four human PDE IV isoforms and the rat PDE IV-C isoform sequences are depicted in Fig. 2.

3.2. *Functional and pharmacological characterization of the PDE IV-C isoenzyme*

The human PDE IV-C was introduced into a yeast strain lacking endogenous PDE activity. PDE deficient yeast is sensitive to heat shock because of an inhibition of cell proliferation by increased cAMP levels formed due to the activation of adenylate cyclase during heat shock. The exogenous PDE IV-C gene restores the heat shock resistance of the yeast cells (Fig. 3) by reducing the elevated level of cAMP [24].

The recombinant hPDE IV-C protein was partially purified from the expressing yeast and assayed for cAMP hydrolysis activity and sensitivity to PDE IV specific inhibitors. As shown in Fig. 4, the recombinant protein showed a K_m of 1.5 ± 0.3 μM for cAMP hydrolysis and a relative V_{max} of 37 ± 5.2 nmol/min/mg (mean ± S.E.M. from 5 experiments) (Fig. 4a). The rank order of potency for PDE inhibitors was Rolipram > Denbufylline > Ro 20–1724 > IBMX (3-Isobutyl-1-methylxan-

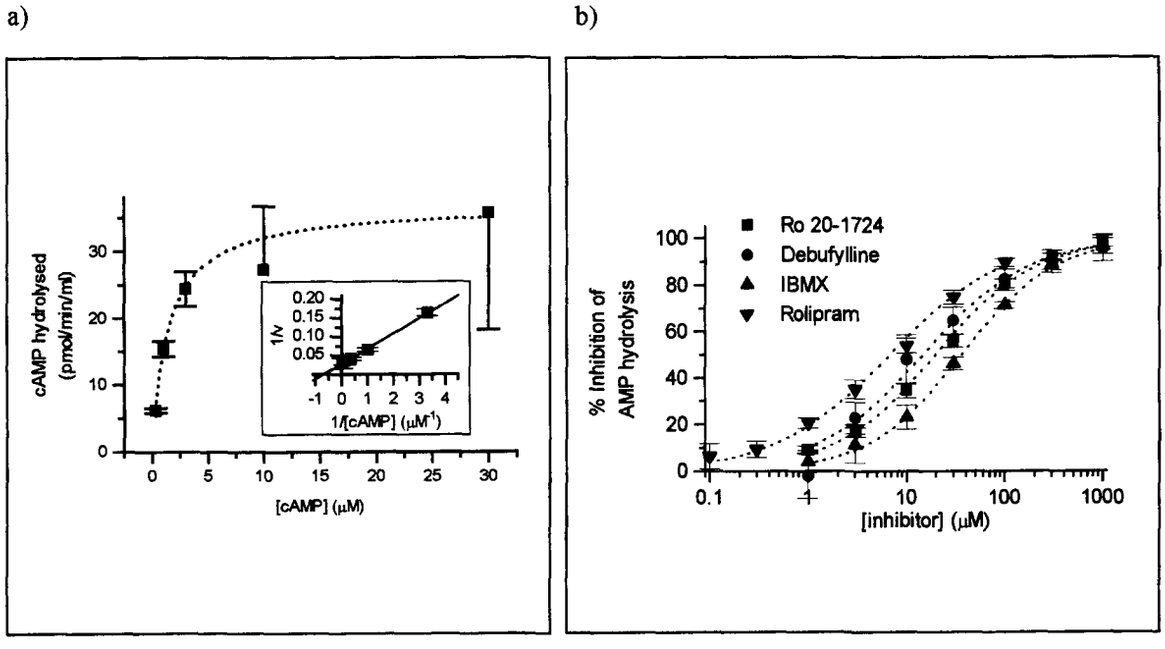

Fig. 4. Pharmacological analysis. (a) Kinetic analysis of the hydrolysis of [^{3}H]cAMP from supernatant in yeast extracts containing the recombinant hPDE IV-C enzyme. K_m and V_{max} values were determined by standard methods (five measurements for each data point, presented as mean ± S.E.M.). The straight line of the Lineweaver-Burk plot (inset) indicates simple Michaelis-Menten-type kinetics. (b) Effect of different inhibitors on enzyme activity in extracts from yeast cells containing the recombinant hPDE IV-C. Curve fitting was performed under the assumption that excessive concentrations of inhibitors completely block the enzyme.

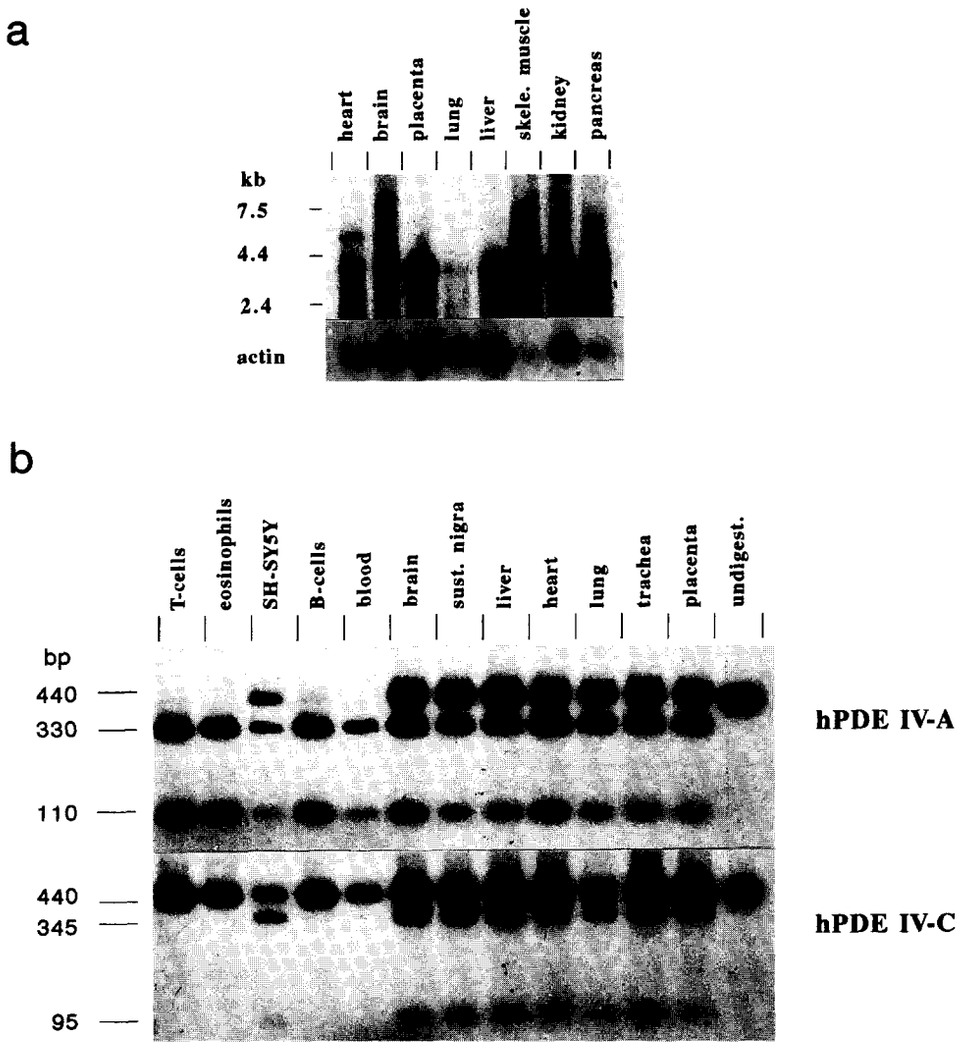

Fig. 5. Tissue distribution of hPDEIV-C. (a) Northern blot analysis was performed with 2 μg of poly(A)$^+$ RNA from various human tissues. A hPDE IV-C specific probe was used. (b) RT-PCR comparison of the two hPDE IV isoforms A and C in different tissues and cell lines. The PCR products (440 bp) obtained with the primer pair PE3/PE4 were cleaved with either *Bst*XI or *Eco*RV to distinguish between the two hPDE IV isogenes A and C. *Bst*XI cleaved specifically in the PCR fragments derived from the hPDE IV-A cDNA (330 bp +110 bp), *Eco*RV cleaved PCR fragments amplified from hPDE IV-C cDNA (345 bp + 95 bp).

thine) with IC_{50} of 6.6 ± 0.6, 14.6 ± 3.1, 19.6 ± 1.4 and 36.2 ± 2.8 μM, respectively (Fig. 4b).

3.3. Tissue distribution of the human PDE IV-C

PDE IV distribution was determined by Northern blot hybridization (Fig. 5a) and reverse polymerase chain reaction (RT-PCR; Fig. 5b). Northern blot analysis of RNAs isolated from human tissues revealed a major band of 4.2 kb in all tissues and a minor band of 5.7 kb present only in heart and skeletal muscle (Fig. 5a). A very weak PDE IV-C signal was observed in lung. Various human tissues and cell lines were also tested by RT-PCR demonstrating that the PDE IV cDNA was detectable in all tissues analyzed with the exception of blood (Fig. 5b). It is particularly interesting that in different cell types of the immune system like lymphocytes or eosinophils we could not observe a specific signal. In the tested human neuronal cell lines SH-SY5Y (Fig. 5b) and SH–N–SH (data not shown) we found a specific restriction of the PCR fragment with EcoRV indicating the expression of hPDE IV-C in this cells.

4. Discussion

We have isolated a full-length cDNA clone for the human phosphodiesterase type IV-C isoform (PDE IV-C) from substantia nigra and SH-SY5Y cell libraries. Pharmacological analysis of the recombinant enzyme expressed in yeast revealed that the hPDE IV-C enzyme has typical PDE IV characteristics [17,24] like low K_m cAMP hydrolysis (1.5 μM) and rolipram sensitivity (IC_{50} = 6.6 μM). The pharmacological properties of the recombinant hPDE IV-C showed small differences compared to other known human PDE IV isoforms [15,16,17,28,29]. hPDE IV-A, B and D showed higher K_ms (18, 8 and 7.2 μM, respectively) but lower rolipram sensitivities with IC_{50} of 0.5, 0.4 and 0.18 μM, respectively.

The amino acid sequence contains all conserved motifs described for PDE IV isoforms [17]. The catalytic domain is more than 80% homologous to those of the other human PDE IV enzymes. Upstream of this catalytic domain, two conserved domains with unknown functions, UCR1 and UCR2

(UCR = upstream conserved region), have been described. Recently it has been reported that one of the rat PDE IV isoforms (rPDE IV-D) is rapidly activated by cAMP-dependent phosphorylation even in the absence of protein synthesis [30]. The presence of a cAMP-dependent protein kinase phosphorylation motif in UCR1 suggests that this region may be involved in short-term regulation of PDE IV activity. This requires additional analysis, however, since a further PKA site is present in the catalytic domain.

Genomic analysis revealed that hPDE IV-C is encoded by a single copy gene (data not shown). The previously published partial human PDE IV-C sequence was mapped to human chromosome 19 [31]. Northern blotting indicated the presence of a 4.2 kb RNA in all tissues and a tissue-specific occurrence of an additional 5.7 kb RNA. This RNA may be produced by differential splicing or alternative use of polyadenylation sites or promoters. Differential RNA splicing has been described for other rat and human PDE IV isoforms [17,27,28,32]. It has been reported for the rat PDE IV-A (RD1) gene that splice variants direct the enzyme to distinct subcellular localizations. A short leader sequence in the N-terminus of the rat PDE IV-A directs the enzyme to the membrane [6]. Further investigation will elucidate if the 5.7 kb RNA serves a similar function.

The hPDE IV-C tissue distribution analysis revealed a distinct but overlapping pattern compared to other isoforms [17,18]. In contrast to the hPDE IV-A, -B and -D, hPDE IV-C is absent in cells of the immune system [18]. We found expression of the human PDE IV-C isogene mRNA in total brain and in substantia nigra. Surprisingly, it is almost absent in these regions of the rat brain [18,33]. This difference between rat and human may indeed be caused by species-specific expression patterns. It could, however, also be explained if another, as yet unknown rat PDE IV isogene homologous to the human PDE IV enzyme described here existed. There is, however, no experimental evidence of yet another isoform.

The use of recombinant proteins will be useful to find isoform specific inhibitors which will help to assign the various physiological functions of PDE IV to specific enzyme subtypes. For medical use, subtype-specific inhibitors may reduce possible undesirable side effects occurring with unselective PDE IV inhibitors like rolipram and may be helpful for the treatment of CNS diseases like depression or Parkinson's disease or peripheral diseases like asthma or atopic dermatitis.

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