

# 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<sup>2+</sup>/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<sup>+</sup> [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'-GTCCAAACACATGACCCCTCTGGCTGACCTG-3'; PE 2: 5'-GCAGGAGGGAGCTGATTGCTGGATGAAG-3'; PE 3: 5'-TCAGAGCTGGCGCTTATGTAC-3'; PE 4: 5'-CCGTATGCTTGTCACACAT-3'; PE 5: 5'-TCAAGCTGCTGCAGCAGAG-3'; PE 6: 5'-GAGTCCTTCTGTACCGTCA-3'; PE 7: 5'-GACTGGA-GCCTGCATAATCCG-3'; PE 8: 5'-AGGTCAAAGCGCCTGC-AGGAGG-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  $\lambda$ -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  $\mu$ g poly(A)<sup>+</sup> 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  $4 \times 10^5$  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  $\mu$ g DNA was used for a PCR analysis which specifically detected the cDNA clone isolated from the substantia 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  $\mu$ g poly(A)<sup>+</sup> 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<sub>4</sub> pH 7.2, 7% SDS, 1% BSA, 50% formamide at 42°C. The blot was washed under stringent conditions with 0.1  $\times$  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  $\mu$ l in the presence of 1  $\mu$ Ci [ $\alpha$ -<sup>32</sup>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 *EcoRV* which cuts specifically in the hPDE IV-C cDNA, or *BstXI*

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-) promoter and 3-phosphoglycerate kinase (PGK-) terminator spaced by a multiple cloning site. The hPDE IV-C cDNA was ligated into the *BamHI/SalI* restriction sites behind the GPD promoter. 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].

1	CCA GTC TGC GGA CCG TTC GGA GCA ACG TGG CGG CCC TTG CCC GCC AGC AAT GCC TAG GAG CAG CCA AGC AGG GAC CCG TCG GAA ACC CTT CAT CCA GCC	99
100	TTT CTC TGG CGC M E N L G V G E G A E A C S R L S R S R G R H S M T R A P	29
101	ATG GAG AAC CTG GGG GTC GGC GAA GGG GCA GAG GCT TGC AGC AGG TTG AGT CGC TCT CGC GGC CAC AGC ATG ACC AGA GCC CCG	198
30	K H L W R Q P R R P R R I R I CAG CGC TTC TAT TCG DAT CCG GAC AAG TCC GCG GGC TGC CGC GAG AGG GAC CTG AGC	62
199	AAG CAC CTG TGG CCG CAA CCC CGG CGC CCC ATC CGC ATC CAA CAG CGC TTC TAT TCG DAT CCG GAC AAG TCC GCG GGC TGC CGC GAG AGG GAC CTG AGC	297
63	P R P P E L R K S R L S W P V S S C R R R F D L E N G L S C G G R R A A L	95
298	CCG CGG CGC GAG CTC AGG AAG TCG CGG CTC TCC TCC CAG CGC TTT GAC CTG GAA AAT GGG CTC TCG TGT GGG AGG AGG ACC CTG	396
96	D P Q S C P G L G R I M Q A P V P H S Q R E S F L Y R S D S D Y	128
397	GAC CCT CAG TCC AGC CCT GGC CTG GGC CGG ATT ATG CAG GCT CCA GTC CCG CAC AGC CAG CGG CGC GAG TCC TTC CTG TAC CGC TCA GAT AGC GAC TAT	495
129	E L S P K A M S R N S S V A S D L H G C E D M I V T P F A Q V L A S	161
496	GAA CTC TCG CCC AAG GCC ATG TCT CGG AAC TCC TCT GTG GCC AGC GAC CTA CAT GGA GAG GAC ATG ATT GTG ACG CCC TTT GCC CAG GTC CTG GCC AGT	594
162	L R T V T R S N V A A L A C R Q Q C L G A A A K Q G P V G N P S S N Q	194
595	CTG CGG ACC GTT CGG AGC AAC GTG GCG GCC CTT GCC CGC CAA TGA GCA GCA GCC AAG CAG GGA CCC GTT GGA AAC CCT TCA TCC AGC AAT CAG	693
195	L P P C A E D T G Q K L A L E T L D E L D W C L D Q L E T L Q T R H	227
694	CTC CCT CCA GAG GAG ACG GGG CAG AAG CTG GCA TTG GAG ACG CTA GAC CAG CTG TGG TGC CTG GAT CAG TTG GAG ACG CTG CAG ACC CGG CAC	792
228	S V G E M A S N K F K R A T L N R E L T H L S E T S R N Q V S E	260
793	TCG GTG GGG GAG ATG GCC TCC AAC AAG TTC AAG CGG ATC CTG AAC CGG GAG TTG ACC CAG CTG TCC GAA ACC AGC CGC TCC GGG AAC CAG GTG TCC GAG	891
261	Y I S R T F L G D Q Q T E V E L P K V G C T A E E A P Q P M S R I S G L	293
892	TAC ATC TCC CGT ACC TTC CTG CAG CAG ACC GAG GTG GAG CTG CCC AAG GTG ACC GCT GAG GGC CCA CAG CCC ATG TCC CGG ATC AGT GGC CTA	990
294	H G L C H S A S L S A T V C P R F G V Q T D Q E E Q L A K E L A E D	326
991	CAT GCG CTC TGC CAG AGT GCC ACG CTC TCC TCA GCG ACT GTG CCA CGC TTT GGG GTC CAG ACT GAC CAG GAG GAG CAA CTG GCC AAG GAG CTA GAA GAC	1089
327	T N K W G L D V F K V A D V S G N R P L T A I I F S I F Q E R D L	359
1090	ACC AAC AAG TGG GGA CTT GAT GTG TTC AAG GTG GCG GAC GTA AGT GGG AAC CGG CCC CTC ACA GCT ATC ATA TTC AAG ATT TTT CAG GAG CGG GAC CTG	1188
360	L K T F I P A D T F Y D L M L H Y H N V A Y H N A N V A Y H N A	392
1189	CTG AAG ACA TTC CAG ATC CCA GCA GAC ACA CTG GCC ACC TAC CTG CTG ATG CTG GAG GGT CAC TAC CAC GCC AAT GTG GCC TAC CAC AAC AGC CTA CAT	1287
393	A A D V A Q S T H V L L A T P A L E A V F T D L E I L A A L F A S	425
1288	GCC GCC GAG GTG GCC CAG TCC ACG CAT GTG CTG CTG GCT ACG CCC GCC CTC GAG ACT GTG TTC ACA GAC TTG GAA ATC CTG GCT GCC CTC TTT GCA AGC	1386
426	A I H D V D H P G V S N Q F L I N T N S D V A L M Y N D A S V L E	458
1387	GCC ATC CAC GAG GTG GAT CAT CCT GGG GTC TCC AAC CAG TTG ATT CAG ACC AAG TCA GAC GTG GCG CTT ATG TAC AAC GAC GCC TCG GTG CTG GAG	1485
459	N H H L A V G C K L L A Q E T C D I F Q N L S A K Q N L S R M	491
1486	AAC CAT CAC CTG GCT GTG GGC TTC AAG CTG CTG CAG GCA GAG AAC TGC GAT ATC TTC CAG AAC CTC AGC GCC AAG CAG CAG CTG AGT CTG CGC AGG ATG	1584
492	V I D M V L A T D M S K H M N L L A D L K T M V E T K K V T A S L G	524
1585	GTC ATT CAG ATG GTG CTG GCC ACA ATG ATG TCC AAA CAC ATG AAC CTC CTG GCC GAG CAC CTC AAG ACC ATG GTG GAG ACC AAG AAG GTG ACA AGC CTC GGT	1683
525	V L L L D N Y S D R I Q V L Q N L V H C A D L S N P T K P L P L Y	557
1684	GTC CTC CTC CTG GAC TAT TCC GAC CGA ATC CAG GTC TTG CAG AAC CTG GTG CAC TGT GCT CAG CTG AGC AAC CCC ACC AAC CCG CTG CCC CTG TAC	1782
558	R Q W T D R I M A E F Q G D R E C E S G L D I S P M C D K H T	590
1783	CGC CAG TGG ACG GAC CGC ATC ATG GCC GAG TTC TTC CAG CAG GGA GAC CGC GAG CGT GAG TCG GGC CTG GAC ATC AGT CCC ATG TGT GAC AAG CAT ACG	1881
591	A S V E K S Q V G F I D Y I A H P L W E T W A D L V H P D A Q D L	623
1882	GCC TCA GTG GAG AAC TCC CAG GTG GGT TTC ATT GAC TAC ATT GCT CAC CCA CTG TGG GAG ACT TGG GCT GAC CTG GTC GAC CCA GAT GCA CAG GAC CTG	1980
624	L D L L E D A N R E W Y Q S K I P C R S P S D L L N P E R D G P D R F	656
1981	CTG GAC ACG CTG GAC AAT CGA GAG TGG TAC CAG AGC AAG ATC CCC CGA AGT CCC TCA ACC AAC CCC GAG CGG GGG CCT GAG AGA TPC	2079
657	Q F E L T L E E A E E D E E E E G E E T A L A K E A L E L P	689
2080	CAG TTT GAA CTG ACT CTG GAG GAG GCA GAG GAA GAG GAT GAG GAG GAA GAG GAG GGG GAA GAG ACA GCT TTA GCC AAA GAG GCC TTG GAG TTG CCT	2178
690	D T E L L S P E A G P D P G D L P L D N Q R T	712
2179	GAC ACT GAA CTC CTG TCC CCT GAA GCC GGC CCA GAC CCT GGG GAC TTA CCC CTC GAC AAC CAG AGG ACT TAG GGC CAG CCC TGC GTG AAC TGC AGG GGC	2277
2278	AAT GGA TGG TAA AGC CCT TTG GCT CTT GGC AGG CAG ACT TTC CAG GAA GAG GCT CCA TGT GGC TCC TGC TTC ACT TTC CCA CCC ATT TAG GGA GAC AAT	2376
2277	CAA GCT CTT AGT TAT AGG TGG CTC CCA GGG TCT AAT TGG AGG CAC CTG GCT GGG GTC CAC TCT GAC CCT AGA CTT GCC TAA AAG AGC TCT CTA AGG GGC	2475
2476	AGC CTC TTA CGA TGC CCT GGT TTC TTT CTC CTG GGC TTC TAT CCC TGT GAG AGG TGC TGT CTG CTG GAG CCT CTA GTC CAC CCT CTC CAG TGG TCA	2574
2575	CTC TTG AGT CAC ATC TGT CAC TTA ATT TCC TTT ATC AAA TAT TTA TTG CTC ATC TAC TTC GGG CCA GCT TTC TGC CTC TGT AGT AGC CCT GCA	2673
2674	CAA AGG GTG GGG AGT CAG GAG ACC ATC CCA AAG GCA TCT CCC TGT CTT CTT CTA CCA AGC GGC TCT CTG CAA GAG CAT GGA AAT GTG AGT GGG GAA AAT	2772
2773	TTT CAG CAC CAA AGC TTC ACT CAT ACC CAG TTT TGT TTC TGA AAC TAC GGT AGG GGG CAG GAA GAG GAG CAG AAA AGA AGG GCT GGG CAA GGC ATA GTG	2871
2872	GCT TAT GCC TGT AAT CCC GGT ACT TTG GGA GGC TGA GGT GGG AGG ACT CTT TAA GCT CAG GAG TTT GAG ACC AGC CTG GGC ATA GCA AGA CCC CCA	2970
2971	CCA TCT CTG AAA AAA AAT AGC CAG GCA TGG TGG TGT GCA CCG TCT GAG AAT CCC AGC TAC TCA GAA GGT TGA GAC AAA GGG GAT CGC TTG AGC CCA GGA	3069
3070	GTT GGA GGC TGA AGA GAG CTA TGA CTG CAT CAC TGC ACT CCA GCC TGG GCA ACA CAG CAA GAT CCT GTC TAA AAA TAA AAA GAA AAG AGA AGG AAA GGA	3168
3169	AAG AGA GGC GGC TCT GAG GGC CAG AGT GGC CCA TGC CTA TAA TCC CAG CAC TTT GGG AGG CTG AGG CAG GTG GAT CAC CTG AGG TTA GGA GTT CGA	3267
3268	GAC CAG CCT GGC CAA CAT GGT GAA ACC CCA TCT CTA CTA AAA ATA CAA AAA TTG GCT GGG CAT GGT GGC GGG TGC CTG TAA TCC CAG CTA CTG GGG AGG	3366
3367	CTG AGG CAG GAG AAT CAC TTG AAT TCA GGA GGT GGA GGT TGC AGT GAG CCG ACA TCA TGC CAC TGC ACT CCA GCC TGG GGC TGA CAG AGC AAG ACA CTG	3465
3466	TCT CAA AAA AGA AAA AAA AAA AAA AAA	3495

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

hPDE IV-C	MENLVGGEA	EACSRLSRSR	GRHSMTRAPK	-HLWRQPRRP	IRIQQRFYSD	PDKSAGCRER	DLSP-RPELR	KSRL-----	-----SWP	VS-----	77
hPDE IV-A	**PPT*PSE	SLSL*PGP*	EGQATLKE*P	*****G**	*****G**	-----ERA*	ERQ*H**IE*	ADAMTSDRP	GLRTTRM**	S*PHGTGTGS	97
hPDE IV-B	**KKSRSVMV	M*DDNVKDYF	ECSLSKSYSS	SSNTLGLDLW	RGRRCSSGNL	Q-LPPLSQRV	SERART**GD	GISR---PTT	LPLTTL*P*IA	I*-----	88
hPDE IV-D	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----MMHVN	6
UCR1											
hPDE IV-C	-----SCRR	FDLENGLSG	RRALDPQSSP	GLGRIMQAPV	P-HSQRRSEF	LYRSDSDYEL	SPKAMSRNSS	VASDLHGDEM	IVTPFAQVLA	SLRTVRSNVA	170
hPDE IV-A	GGAGGG*ST*	*EA***PTPS	PGRSPLD*QA	SP*LVLH*GA	A-T*****	*****DM	***T*****	*T*EA*A*L	*****	***S***F	195
hPDE IV-B	-----TV*QEC	**V***P*P*	*SP***A*S	SA*LVLH*TF	*G*****	*****D*	*****D*	LP*EQ*+D*L	*****	***S***F	183
hPDE IV-D	PFPRRH*WIC	**VD**T*A*	*SP***MT*	*S*L*L*N*F	V*****	*****D*	*****D*	I**I**+D*L	*****	***S***F	105
rPDE IV-C	-----	-----	-----	-----	-----	-----	-----	NS**T*	A*****	*****	37
UCR2											
hPDE IV-C	ALARQQCLGA	AKQGPVGNPS	SSNQLPPAED	TGQKLALETI	DELDMLDQL	ETLQTRHSVG	EMASNKFKRI	LNRELTHLSE	TSRSGNQVSE	YISRIFLDQ	270
hPDE IV-A	S*LTNVVPS	N*RS*L*G*T	PVCKATLS*E	*C*Q*R**	E*****E*	*****M*	*****H**M	*****M*	*****M*	***T***K*	295
hPDE IV-B	TIITNLHGTS	N*RS*AASQP	PVSRVN*Q*E	SY***M**	E*****E*	***I*YR**	*****M*	*****M*	*****M*	***N***K*	283
hPDE IV-D	*T*NL*DRAP	S*RS*MC*QP	*I*KATIT*E	AY***S**	E*****E*	*****S*	*****M*	*****M*	*****M*	F*N***K*	205
rPDE IV-C	**HGAGSAT	RQALLGTP*Q	**Q*AA**E	S*LQ**Q**	E*****E*	*****R**	*****M*	*****M*	*****M*	***Q***V*	137
catalytic domain											
hPDE IV-C	TEVELPKVTA	-----	-----	EEAPQPMRSI	SGLHGLCHSA	SLSSATVPRF	GVQTDQEEQL	AKELEDTNKW	GLDVKFVADV	SGNRPLTAII	350
hPDE IV-A	N***I*SP*M	KEREKQAPR	PRPSQPPEPP	VPHL**C**Q*	T**KK**M**N	*****NNSNI**	**K***L*	*Q***NL**	*****	***S***C**M	395
hPDE IV-B	ND**I*SP*Q	KDREKKK	-----	--KQ*L*TQ*	**VKK**M**S	*****NNTSIS*	**N*EN*DH*	*****L**	*****	***H***C**M	368
hPDE IV-D	H***I*SP*Q	KEKEKKK	-----	-----R**Q*	**VKK**M**S	*****TNSSI**	**K*E**DV*	*****V**	*****	***V***V**M	288
rPDE IV-C	A***APPT	-----	-----	--DHPWMAQ	ITLGRKSCHT	*****PT*AI**	*****	*****	*****	*****EL	216
catalytic domain											
hPDE IV-C	FSIFQERDLL	KTFQIPADTL	ATYLLMLEGH	YHANVAYHNS	LHAADVAQST	HVLLATPALE	AVFTDLEILA	ALFASAIHDV	DHPGVSNQFL	INTNSDVALM	450
hPDE IV-A	YM*****	*K*R**V**M	V**M*T**D*	**D*****	*****L*	*****S**D	*****D	*****A*	*****	*****EL**	495
hPDE IV-B	YA*****	*R*SS**F	I**MMT**D*	**S*****	*****S**D	*****S**D	*****D	*****I**A*	*****	*****EL**	468
hPDE IV-D	HT*****	**K*V**	I**MT**D*	**S*****	*****S**D	*****S**D	*****D	*****I**A*	*****	*****EL**	388
rPDE IV-C	*RVL*****	*****	LR*****	**S*****	*****S**D	*****S**D	*****D	*****I**C*	*****	*****EL**	316
catalytic domain											
hPDE IV-C	YNDASVLENH	HLAVGFKLQ	AENCDFQNL	SAKQRLSLRR	MVIDMVLATD	MSKHMNLAD	LKTMVETKKV	TSGLVLLDN	YSYRIQVLQN	LHVCADLSNP	550
hPDE IV-A	***E*****	*****	ED*****	*KR**Q**K	*****	*****	*****	*****	*****	*****	595
hPDE IV-B	***E*****	*****	E*H*****	TK**QT**K	*****	*****	*****	*****	*****	*****	568
PDE21	-----	*****	*****	*****	*****	*****	*****	*****	*****	*****	89
hPDE IV-D	***S*****	*****	E*****	TK**Q**K	*****	*****	*****	*****	*****	*****	488
rPDE IV-C	***S*****	*****	G*****	T**K**K	*****	*****	*****	*****	*****	*****S	416
catalytic domain											
hPDE IV-C	TKPLPLYRQW	TDRIMAEFFQ	QGDRERESGL	DISPMCKDT	ASVEKSQVGF	IDYIAHPLWE	TWADLVHPDA	QDLLDTLEDN	REWYQSKPR	SPSDDLTPNER	650
hPDE IV-A	***E*****	*****	*****R**M	E*****	*****	*****V*****	*****	*****EI*****	*****D**Y**A**RQ	***PPPEE*S	695
hPDE IV-B	***S*E*****	*****E*****	**K**R**M	E*****	*****	*****V*****	*****Q**	*****	*****N*****M**Q	***PPLDEQN	668
PDE 21	*****	*****	*****R**M	E*****	*****	*****V*****	*****	*****	*****N*****M**Q	*****	189
hPDE IV-D	***Q*****	*****E*****R	*****R**M	E*****N	*****	*****V*****	*****	*****	*****I*****T**Q	***PAPDPE	588
rPDE IV-C	A*****	E*****	*****	*****	*****	*****V*****	*****	E*****	*****RV**C	**PHATG*D*	516
catalytic domain											
hPDE IV-C	DGPRDFQFEL	TLEEAEEDE	EEEEEGEETA	LAKEALELPD	TELLSPEAGP	DPGDLPLDNQ	RT 712	-----	-----	-----	795
hPDE IV-A	R**GHPPLPD	KQFQLEILE*	*****SMAQ	I*CTAQEALT	AQGLSGVEEA	LQATIAEAS	PAQESLEVMA	QEASLEAELE	AVYLTQQAQS	TGSAPVAPDE	795
hPDE IV-B	RDCQGLMEKF	QF*LTLD*ED	*****S*GP*K*GEG	HSYFSSSTKL	CVIDPENRDS	LGCTDIDIAT	EDKSEVDT 736	-----	-----	-----	736
PDE 21	-----	*****	*****	*****	*****	*****	*****	*****	*****	*****	251
hPDE IV-D	E*RGQQTEKF	QF*LTLD*ED	*****S*GP*K*GEG	HSYFSSSTKL	CVIDPENRDS	LGCTDIDIAT	EDKSEVDT 736	-----	-----	-----	736
rPDE IV-C	FKFELTE*V	EE**E*D*RH	536	-----	-----	-----	-----	-----	-----	-----	536
hPDE IV-A	FSSREEFVVA	VSHSSPSALA	LQSPLLPAWR	TLVSEHAPG	LPGLPSTAAE	VEAQREHQAA	KRACSACAGT	FGEDTSALPA	PGGGSGGDP	T 886	886

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 phenylmethylsulphonyl 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 cholamidopropyl 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 ([<sup>3</sup>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 *Ophiophagus hannah*). The unreacted substrate was separated from [<sup>3</sup>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<sub>50</sub> 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

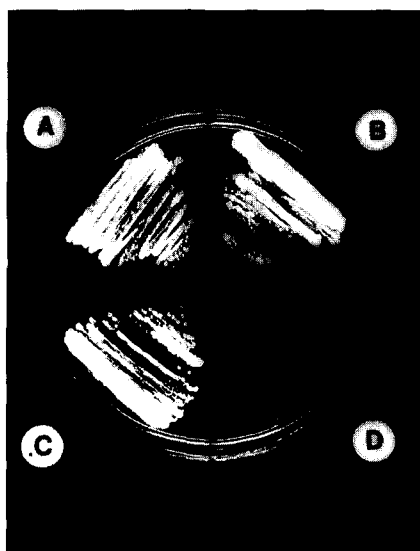


Fig. 3. Heat shock assay for functional hPDE IV-C expression in yeast. A/C = PDE deficient yeast containing pYEMS IV-C without/with heat shock; B/D = PDE deficient yeast without/with heat shock. Cells were grown after the heat shock (3 min, 55°C) for 16 h at 30°C in 1 ml of liquid medium. 2  $\mu$ l 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  $1.5 \pm 0.3 \mu\text{M}$  for cAMP hydrolysis and a relative  $V_{\max}$  of  $37 \pm 5.2 \text{ nmol/min/mg}$  (mean  $\pm$  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-

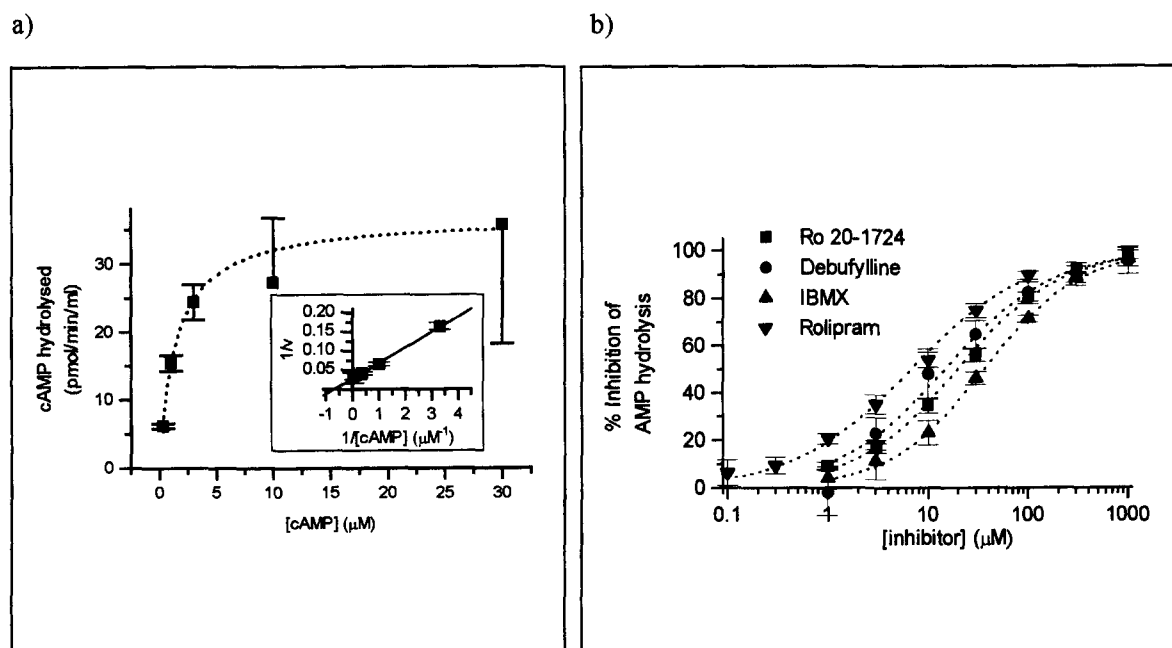


Fig. 4. Pharmacological analysis. (a) Kinetic analysis of the hydrolysis of [ $^3\text{H}$ ]cAMP from supernatant in yeast extracts containing the recombinant hPDE IV-C enzyme.  $K_m$  and  $V_{\max}$  values were determined by standard methods (five measurements for each data point, presented as mean  $\pm$  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.

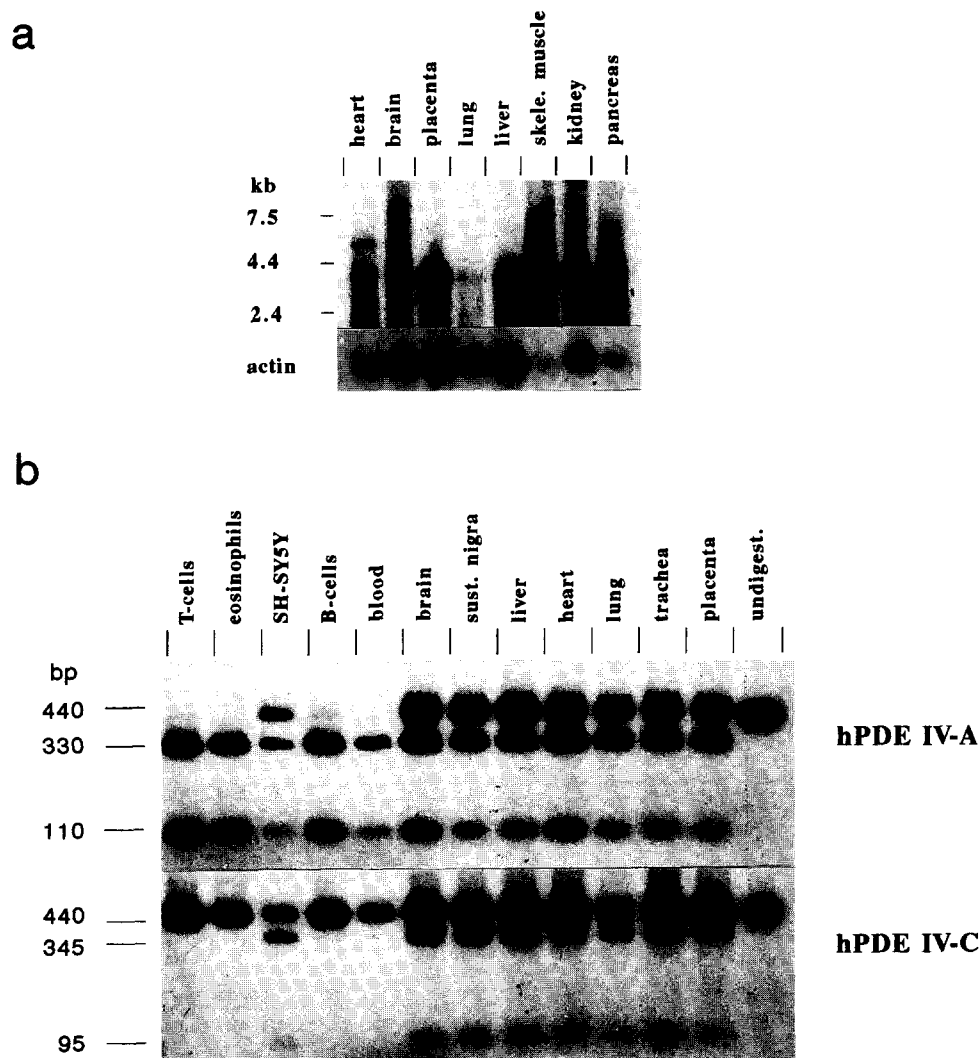


Fig. 5. Tissue distribution of hPDE IV-C. (a) Northern blot analysis was performed with 2  $\mu$ g of poly(A)<sup>+</sup> RNA from various human tissues. A hPDE IV-C specific probe was used. (b) RT-PCR comparison of the two hPDE IV isoforms A and C in different tissues and cell lines. The PCR products (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 isoforms 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  $6.6 \pm 0.6$ ,  $14.6 \pm 3.1$ ,  $19.6 \pm 1.4$  and  $36.2 \pm 2.8$   $\mu$ M, 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 *Eco*RV 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  $\mu$ M) and rolipram sensitivity ( $IC_{50} = 6.6$   $\mu$ M). 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  $K_m$ s (18, 8 and 7.2  $\mu$ M, respectively) but lower rolipram sensitivities with  $IC_{50}$  of 0.5, 0.4 and 0.18  $\mu$ M, 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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