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Cloning and Expression of cDNA for a Human Low-K_m, Rolipram-Sensitive Cyclic AMP Phosphodiesterase

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We have isolated cDNA clones representing cyclic AMP (cAMP)-specific phosphodiesterases (PDEases) from a human monocyte cDNA library. One cDNA clone (hPDE-1) defines a large open reading frame of ca. 2.1 kilobases, predicting a 686-amino-acid, ca. 77-kilodalton protein which contains significant homology to both rat brain and *Drosophila* cAMP PDEases, especially within an internal conserved domain of ca. 270 residues. Amino acid sequence divergence exists at the NH_2 terminus and also within a 40- to 100-residue domain near the COOH-terminal end. hPDE-1 hybridizes to a major 4.8-kilobase mRNA transcript from both human monocytes and placenta. The coding region of hPDE-1 was engineered for expression in COS-1 cells, resulting in the overproduction of cAMP PDEase activity. The hPDE-1 recombinant gene product was identified as a low-K_m cAMP phosphodiesterase on the basis of several biochemical properties including selective inhibition by the antidepressant drug rolipram. Known inhibitors of other PDEases (cGMP-specific PDEase, cGMP-inhibited PDEase) had little or no effect on the hPDE-1 recombinant gene product. Human genomic Southern blot analysis suggests that this enzyme is likely to be encoded by a single gene. The presence of the enzyme in monocytes may be important for cell function in inflammation. Rolipram sensitivity, coupled with homology to the *Drosophila* cAMP PDEase, which is required for learning and memory in flies, suggests an additional function for this enzyme in neurobiochemistry.

Cyclic nucleotide phosphodiesterases (PDEases) consist of a complex family of enzymes whose function is to catalyze the hydrolysis of 3′,5′-cyclic nucleotides into 5′-nucleoside monophosphates. PDEases play a central role in signal transduction, regulating cellular concentrations of cyclic nucleotides, which, in turn, are responsible for mediating the cellular response to extracellular signals such as hormones and neurotransmitters. Many different forms of PDEases have been reported, each exhibiting a unique set of regulatory effectors and kinetic properties. The mammalian PDEases have been classified into multiple isozyme families, distinguished on the basis of substrate affinity and specificity, cofactor requirements, and selective inhibition by various compounds (3, 6). These isozyme families include (i) the Ca^{2+} calmodulin-dependent PDEases, (ii) the cyclic GMP (cGMP)-specific PDEases, (iii) the cGMP-stimulated PDEases, (iv) the cGMP-inhibited PDEases, and (v) the cAMP-specific PDEases (3, 6).

Until recently, our knowledge of the mode of regulation of this diverse class of enzymes was limited to what could be learned from biochemical studies with protein isolated from natural sources. Molecular genetic approaches have led to the isolation of genes for both the low- and high-affinity cAMP PDEases from *Saccharomyces cerevisiae* (*PDE1* and *PDE2*, respectively) (23, 28, 39), the gene for the secreted cAMP-specific PDEase from *Dictyostelium discoideum* (19), the gene for a low-K_m (high-affinity) cAMP-specific PDEase from *Drosophila melanogaster* (dunce$^+$ or *dnc*$^+$) (7), cDNA clones for the bovine and human retinal cGMP-specific PDEases (α subunit) (24, 25), and cDNA clones for rat brain and testis low-K_m cAMP-specific PDEases (10, 13, 17, 32). With the exception of one of the yeast and the slime mold PDEases, significant amino acid sequence homology exists among these enzyme species (3, 10, 13, 17). In fact, several of the rat PDEase cDNAs were cloned by virtue of their DNA sequence homology to the *Drosophila dnc*$^+$ gene (13, 32), a gene required for normal memory of learned information in flies (15). The isolation of four distinct rat cAMP PDEase cDNA clones (from brain and testis) suggests the existence of a multigene family (10, 13, 32). The possibility that the low-K_m cAMP PDEases play an essential role in central nervous system function has been suggested by biochemical studies showing that some of the enzymes are selectively inhibited by compounds which exhibit antidepressant properties, such as RO 20-1724 and rolipram (3, 18, 38).

We have isolated cDNA clones that represent human PDEases. In this report, we present the DNA sequence of one such clone along with an amino acid sequence comparison with other PDEases, indicating conservation within two internal domains. Furthermore, we demonstrate by expression of the cDNA, followed by isozyme-selective inhibitor studies, that it codes for a low-K_m cAMP-specific PDEase that is sensitive to rolipram. Possible functions and pharmacological significance of this enzyme are discussed.

MATERIALS AND METHODS

Isolation and molecular characterization of cDNA. A ^{32}P-labeled 883-base-pair (bp) *Pst*I fragment, containing the conserved region of a rat brain cDNA clone (RD1) coding for a low-K_m cAMP PDEase (13), was prepared by nick translation (26) and used to screen a granulocyte-macrophage colony-stimulating factor-stimulated human monocyte library constructed in λZAP (Stratagene Inc., La Jolla,

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Calif.). A total of 12 clones were isolated and partially characterized by restriction mapping. DNA sequencing of one 2.6-kilobase (kb) clone (hPDE-1) was performed by the method of Sanger et al. (27) with double-stranded plasmid DNA primed with universal primers or synthetic oligonucleotides (see Fig. 1).

Isolation and analysis of genomic DNA. Human DNA was isolated from liver by standard methods (12). DNA samples were digested with the appropriate restriction enzymes, size fractionated on agarose gels, and transferred to nitrocellulose filters. Both the full-length hPDE-1 cDNA and a small 189-bp internal fragment extending from the second *Pvu*II site to the unique *Xho*I site were labeled with ^{32}P by random priming (Pharmacia, Inc.). Hybridizations were carried out at 65°C in 6× SCP (0.6 M NaCl, 0.18 M Na_2HPO_4, 6 mM EDTA [pH 6.2]) containing 1% *N*-lauroylsarcosine, 10% dextran sulfate, and 45 μg of herring sperm DNA per ml. Filters were washed at 55°C in 0.3× SCP–0.1% sodium dodecyl sulfate (SDS) and autoradiographed.

Isolation and analysis of RNA. Total RNA was extracted from primary human monocytes, human placenta, and U-937 cells (human histiocytic lymphoma, ATCC CRL 1593) by using the guanidinium-cesium chloride method (16, 37), and poly(A)$^+$ RNA was selected by chromatography on oligo (dT)-cellulose (2). RNAs were size fractionated on a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, and probed with a ^{32}P-labeled (26) *Xho*I fragment of hPDE-1 (the second *Xho*I site is located in the polylinker sequence of Bluescript at the 5′ end of the clone [Stratagene]). Hybridizations were carried out under the same conditions stated above, and blots were washed under similar moderately stringent conditions.

Expression in COS-1 cells. The coding region of hPDE-1 was inserted into the COS-1 cell expression vector pRJB4 (a gift from D. Bergsma, SmithKline Beecham Pharmaceuticals), which contains the simian virus 40 origin of replication, plus the Rous sarcoma virus long terminal repeat promoter followed by bovine growth hormone poly(A) sequences. pRJB4 was derived from pRJB2 (5). In one construct (pRJB4-hPDE-Met1), a fragment of hPDE-1 extending from the first ATG to the unique *Nco*I site was generated by a polymerase chain reaction (22) to contain a 5′ *Sal*I site. This fragment was used to replace the *Sal*I (5′ polylinker)-to-*Nco*I piece in the original λZAP clone hPDE-1, creating hPDE-1U. The polymerase chain reaction-generated portion of hPDE-1U was sequenced, and the entire coding region was excised with *Sal*I and *Spe*I (3′ polylinker), the ends were filled in with Klenow polymerase, and the fragment was inserted into the unique *Stu*I site of pRJB4. In the second construct (pRJB4–hPDE-Met48), the 3′ two-thirds of the coding region of hPDE-1, carried on a 1,661-bp *Pvu*II (second site shown in Fig. 1)-to-*Spe*I (3′ polylinker site, filled in with Klenow polymerase) fragment, was first inserted into the *Stu*I site of pRJB4, creating pRJB4–hPDE-3′. Part of the 5′ end was restored by cutting pRJB4–hPDE-3′ with *Hin*dIII (a site in RBJ4 located 5′ to the *Stu*I site), filling in the ends (with Klenow polymerase), digesting with *Nco*I, gel purifying the large vector fragment, and inserting the 976-bp *Sma*I-to-*Nco*I fragment from hPDE-1. The hPDE-1 sequence in pRJB4–hPDE-Met48 begins 47 bp upstream from the methionine 48 residue of the coding region (see Fig. 1).

COS-1 monkey kidney cells were maintained at 37°C under 5% CO_2 in Dulbecco modified Eagle medium containing 10% fetal calf serum, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. At 24 h prior to transfection, cells were plated at a density of 1.8×10^6 cells per 100-mm dish. For transfection, cells were rinsed with PBS (10 mM sodium phosphate, 150 mM NaCl [pH 7.3]) and then incubated for 6 h at 37°C with 25 μg of plasmid DNA (pRJB4–hPDE-Met1, pRJB4–hPDE-Met48, or pRJB4 alone) in 5 ml of Dulbecco modified Eagle medium containing 400 μg of DEAE-dextran per ml, 100 μM chloroquine, and 5% fetal calf serum (20, 31). The transfection medium was aspirated, and the cells were shocked with 10% dimethyl sulfoxide in PBS for 3 min at 25°C. Dishes were rinsed once with PBS and incubated for 3 days at 37°C with Dulbecco modified Eagle medium containing 10% fetal calf serum and antibiotics.

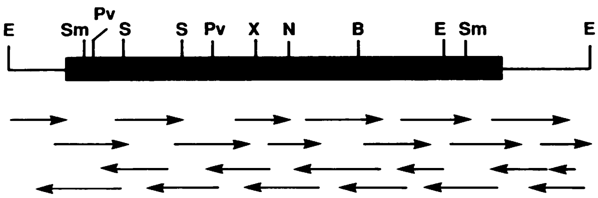
Harvested COS-1 cells were washed with Dulbecco PBS (without Ca^{2+} and Mg^{2+}) and suspended at 5×10^6 to 10×10^6 cells per ml in 10 mM Tris hydrochloride (pH 7.8) containing 250 mM sucrose, 5 mM $MgCl_2$, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*^{1},*N*^{1}-tetraacetic acid (EGTA), 2 mM benzamidine, 100 μM *p*-tosyl-L-lycine chloromethyl ketone, 100 μg of bacitracin per ml, 20 μg of soybean trypsin inhibitor per ml, and 50 μM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication (type 200 cell disruptor; Branson Sonic Power Co., Danbury, Conn.) and centrifuged at $12,000 \times g$ at 4°C for 30 min. Pellets were washed twice and resuspended in the same buffer.

PDEase assays and inhibitor studies. PDEase activity was assayed by a modification (36) of published procedures (11). Reactions were conducted at 30°C in 0.1 ml of standard mixture, containing 50 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, 50 μm [^{14}C]AMP (ca. 400 dpm/nmol), 1 μM [^3H] cAMP (ca. 2,000 dpm/nmol), and enzyme. Background radioactivity was determined by conducting the assay in the absence of enzyme. The same results were obtained with boiled extracts and with samples containing no enzyme. Reactions were terminated by incubation at 100°C for 1 min. To separate cyclic nucleotide substrates from 5′-nucleotide products, 0.5 ml of EQ buffer (0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 8.5] containing 0.1 M NaCl) was added to each sample. The entire sample was then applied to a polyacrylamide-boronate gel column (Affi-Gel 601; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with EQ buffer. Unreacted cyclic nucleotides were eluted with EQ buffer, and the 5′-monophosphate products were eluted with 0.25 M acetic acid. Radioactivity was measured by scintillation spectrometry. Recovery of [^3H]AMP, as determined with the [^{14}C]AMP carrier, was 80 to 90%. All assays were conducted in the linear range of the reaction at which less than 20% of the initial substrate is hydrolyzed. Kinetic assays were controlled such that substrate hydrolysis was limited to 10%. cGMP hydrolysis was assayed as above, with [^3H]cGMP as the substrate.

For determination of V_{max}, K_m, and K_i, the concentration of cAMP or cGMP was varied while the amount of ^3H-labeled cyclic nucleotide per assay was kept constant. Appropriate corrections were made for the changes in specific activity of the substrate. Kinetics were analyzed with computer programs described by Cleland (9), using a nonlinear least-squares regression analysis. Protein content was determined by the method of Lowry et al. (21).

Preparation of antiserum to a GalK–hPDE-1 fusion protein synthesized in *E. coli*. A 1,661-bp *Pvu*II (second site shown in Fig. 1)-to-*Spe*I (3′ polylinker site, filled in with Klenow polymerase) fragment of hPDE-1 was subcloned into the unique *Sma*I site of the *Escherichia coli* expression vector pOTSKF33 (8), creating pOTSKF33–hPDE-1. This plasmid

A



B

-265

CAATGGGCCGACACCATCTCCTGGCCGCAGCCCCCTGGACTCG -221

CAGGCGAGCCCAGGACTCGTGCTGCACGCCGGGGGGCCACCAGCCAGCACCGGGAGTCCTTCCTGTACCGCTCAGACAGCGACTATGACATGTCACCCGAGACCATGTCCCGGAACTCA -102

*** Met Cys Pro Phe 4

TCGGTCACCAGCGAGGCGCACGCTGAAGACCTCATCGTAACACCATTTGCTCAGGTGCTGGCCAGCCTCCGGAGCGTCCGTAGCAACTTCTCACTCCTGACCA ATG TGC CCG TTC 12

Pro Val Thr Thr Val Pro Leu Gly Gly Pro Thr Pro Val Cys Lys Ala Thr Leu Ser Glu Glu Thr Cys Gln Gln Leu Ala Arg Glu Thr 34

CCA GTA ACA ACG GTC CCG CTG GGC GGC CCC ACC CCT GTC TGC AAG GCC ACG CTG TCA GAA GAA ACG TGT CAG CAG TTG GCC CGG GAG ACT 102

Leu Glu Glu Leu Asp Trp Cys Leu Glu Gln Leu Glu Thr Met Gln Thr Tyr Arg Ser Val Ser Glu Met Ala Ser His Lys Phe Lys Arg 64

CTG GAG GAG CTG GAC TGG TGT CTG GAG CAG CTG GAG ACC <u>ATG</u> CAG ACC TAT CGC TCT GTC AGC GAG ATG GCC TCG CAC AAG TTC AAA AGG 192

Met Leu Asn Arg Glu Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu Tyr Ile Ser Thr Thr Phe Leu Asp Lys 94

ATG TTG AAC CGT GAG CTC ACA CAC CTG TCA GAA ATG AGC AGG TCC GGA AAC CAG GTC TCA GAG TAC ATT TCC ACA ACA TTC CTG GAC AAA 282

Gln Asn Glu Val Glu Ile Pro Ser Pro Thr Met Lys Glu Arg Glu Lys Gln Gln Ala Pro Arg Pro Arg Pro Ser Gln Pro Pro Pro Pro 124

CAG AAT GAA GTG GAG ATC CCA TCA CCC ACG ATG AAG GAA CGA GAA AAA CAG CAA GCG CCG CGA CCA AGA CCC TCC CAG CCG CCC CCG CCC 372

Pro Val Pro His Leu Gln Pro Met Ser Gln Ile Thr Gly Leu Lys Lys Leu Met His Ser Asn Ser Leu Asn Asn Ser Asn Ile Pro Arg 154

CCT GTA CCA CAC TTA CAG CCC ATG TCC CAA ATC ACA GGG TTG AAA AAG TTG ATG CAT AGT AAC AGC CTG AAC AAC TCT AAC ATT CCC CGA 462

Phe Gly Val Lys Thr Asp Gln Glu Glu Leu Leu Ala Gln Glu Leu Glu Asn Leu Asn Lys Trp Gly Leu Asn Ile Phe Cys Val Ser Asp 184

TTT GGG GTG AAG ACC GAT CAA GAA GAG CTC CTG GCC CAA GAA CTG GAG AAC CTG AAC AAG TGG GGC CTG AAC ATC TTT TGC GTG TCG GAT 552

Tyr Ala Gly Gly Arg Ser Leu Thr Cys Ile Met Tyr Met Ile Phe Gln Glu Arg Asp Leu Leu Lys Lys Phe Arg Ile Pro Val Asp Thr 214

TAC GCT GGA GGC CGC TCA CTC ACC TGC ATC ATG TAC ATG ATA TTC CAG GAG CGG GAC CTG CTG AAG AAA TTC CGC ATC CCT GTG GAC ACG 642

Met Val Thr Tyr Met Leu Thr Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His Asn Ser Leu His Ala Ala Asp Val Leu Gln Ser 244

ATG GTG ACA TAC ATG CTG ACG CTG GAG GAT CAC TAC CAC GCT GAC GTG GCC TAC CAT AAC AGC CTG CAC GCA GCT GAC GTG CTG CAG TCC 732

Thr His Val Leu Leu Ala Thr Pro Ala Leu Asp Ala Val Phe Thr Asp Leu Glu Ile Leu Ala Ala Leu Phe Ala Ala Ala Ile His Asp 274

ACC CAC GTA CTG CTG GCC ACG CCT GCA CTA GAT GCA GTG TTC ACG GAC CTG GAG ATT CTC GCC GCC CTC TTC GCG GCT GCC ATC CAC GAT 822

Val Asp His Pro Gly Val Ser Asn Gln Phe Leu Ile Asn Thr Asn Ser [Glu Leu Ala Leu Met Tyr Asn] Asp Glu Ser Val Leu Glu Asn 304

GTG GAT CAC CCT GGG GTC TCC AAC CAG TTC CTC ATC AAC ACC AAT TCG [GAG CTG GCG CTC ATG TAC AAC] GAT GAG TCG GTG CTC GAG AAT 912

His His Leu Ala Val Gly Phe Lys Leu Leu Gln Glu Tyr Asn Cys Asp Ile Phe Gln Asn Leu Ser Lys Arg Gln Arg Gln Ser Leu Arg 334

CAC CAC CTG GCC GTG GGC TTC AAG CTG CTG CAG GAG TAC AAC TGC GAC ATC TTC CAG AAC CTC AGC AAG CGC CAG CGG CAG AGC CTA CGC 1002

Lys Met Val Ile Asp Met Val Leu Ala Thr Asp Met Ser Lys His Met Thr Leu Leu Ala Asp Leu Lys Thr Met Val Glu Thr Lys Lys 364

AAG ATG GTC ATC GAC ATG GTG CTG GCC ACG GAC ATG TCC AAG CAC ATG ACC CTC CTG GCT GAC CTG AAG ACC ATG GTG GAG ACC AAG AAA 1092

Val Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln Val Leu Arg Asn Met Val His Cys Ala Asp Leu Ser Asn 394

GTG ACC AGC TCA GGG GTC CTC CTG CTA GAT AAC TAC TCC GAC CGC ATC CAG GTC CTC CGG AAC ATG GTG CAC TGT GCC GAC CTC AGT AAC 1182

Pro Thr Lys Pro Leu Glu Leu Tyr Arg Gln Trp Thr Asp Arg Ile Met Ala Glu Phe Phe Gln Gln Gly Asp Arg Glu Arg Glu Arg Gly 424

CCC ACC AAG CCG CTG GAG CTG TAC CGC CAG TGG ACA GAC CGC ATC ATG GCC GAG TTC TTC CAG CAG GGT GAC CGA GAG CGC GAG CGT GGC 1272

Met Glu Ile Ser Pro Met Cys Asp Lys His Thr Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val His Pro Leu Trp 454

ATG GAA ATC AGC CCC ATG TGT GAC AAG CAC ACT GCC TCC GTG GAG AAG TCT CAG GTG GGT TTT ATT GAC TAC ATT GTG CAC CCA TTG TGG 1362

Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Glu Ile Leu Asp Thr Leu Glu Asp Asn Arg Asp Trp Tyr Tyr Ser Ala Ile Arg 484

GAG ACC TGG GCG GAC CTT GTC CAC CCA GAT GCC CAG GAG ATC TTG GAC ACT TTG GAG GAC AAC CGG GAC TGG TAC TAC AGC GCC ATC CGG 1452

Gln Ser Pro Ser Pro Pro Pro Glu Glu Glu Ser Arg Gly Pro Gly His Pro Pro Leu Pro Asp Lys Phe Gln Phe Glu Leu Thr Leu Glu 514

CAG AGC CCA TCT CCG CCA CCC GAG GAG GAG TCA AGG GGG CCA GGC CAC CCA CCC CTG CCT GAC AAG TTC CAG TTT GAG CTG ACG CTG GAG 1542

Glu Glu Glu Glu Glu Glu Ile Ser Arg Ala Gln Ile Arg Cys Thr Ala Gln Glu Ala Leu Thr Glu Gln Gly Leu Ser Gly Val Glu Glu 544

GAG GAA GAG GAG GAA GAA ATA TCA AGG GCC CAG ATA CGG TGC ACA GCC CAA GAG GCA TTG ACT GAG CAG GGA TTG TCA GGA GTC GAG GAA 1632

Ala Leu Asp Ala Thr Ile Ala Trp Glu Ala Ser Pro Ala Gln Glu Ser Leu Glu Val Met Ala Gln Glu Ala Ser Leu Glu Ala Glu Leu 574

GCT CTG GAT GCA ACC ATA GCC TGG GAG GCA TCC CCG GCC CAG GAG TCG TTG GAA GTT ATG GCA CAG GAA GCA TCC CTG GAG GCC GAG CTG 1722

Glu Ala Val Tyr Leu Thr Gln Gln Ala Gln Ser Thr Gly Ser Glu Pro Val Ala Pro Asp Glu Phe Ser Asn Arg Glu Glu Phe Val Val 604

GAG GCA GTG TAT TTG ACA CAG CAG GCA CAG TCC ACA GGC AGT GAA CCT GTG GCT CCG GAT GAG TTC TCG AAC CGG GAG GAA TTC GTG GTT 1812

Ala Val Ser His Ser Ser Pro Ser Ala Leu Ala Leu Gln Ser Pro Leu Leu Pro Ala Trp Arg Thr Leu Ser Val Ser Glu His Ala Pro 634

GCT GTA AGC CAC AGC AGC CCC TCT GCC CTG GCT CTT CAA AGC CCC CTT CTC CCT GCT TGG AGG ACC CTG TCT GTT TCA GAG CAT GCC CCC 1902

Gly Leu Pro Gly Leu Pro Ser Thr Ala Ala Glu Val Glu Ala Gln Arg Glu His Gln Ala Ala Lys Arg Ala Cys Ser Ala Cys Ala Gly 664

GGC CTC CCG GGC CTC CCC TCC ACG GCG GCC GAG GTG GAG GCC CAA CGA GAG CAC CAG GCT GCC AAG AGG GCT TGC AGT GCC TGC GCA GGG 1992

Thr Phe Gly Glu Asp Thr Ser Ala Leu Pro Ala Pro Gly Gly Gly Gly Ser Gly Gly Asp Pro Thr *** 686

ACA TTT GGG GAG GAC ACA TCC GCA CTC CCA GCT CCT GGT GGC GGG GGG TCA GGT GGA GAC CCT ACC TGATCCCCAGACCTCTGTCCCTGTTCCCCTC 2086

CGCTCCTCCCCTCACTCCCCTGCTCCCCCGACCACCTCCTCCTCTGCCTCAAAGACTCTTGTCCTCTTGTCCCTCCTGAGAAAAAAGAAAACGTAAAAGTGGGGTTTTTTCTGTTTTC 2205

TTTTTTTCCGCTTGGCCCCTGCCCCCCACCCACGGGGGCTTTTTTTTTGGAGGTGGGGGCTGGGGAATGAGGGGCTGAGGTCCGGAAAAGTTTATTTTTTTGAATTTTAATTGTAACA 2324

TTTTTAGAAAAAGAACAAAAAAAAAAG 2351

contains a portion of the hPDE-1 sequence fused to the first 56 amino acids of *E. coli* galactokinase (GalK) (14). Expression is driven by the nalidixic acid-inducible λ phage promoter (p_L). pOTSKF33–hPDE-1 was introduced into cells of strain AR120, and protein expression was induced by the addition of 60 μg of nalidixic acid per ml for 4 h. Cells were lysed, and GalK–hPDE-1 fusion protein was gel purified by the method of Aldovini et al. (1). Approximately 100 μg of this purified fusion protein was injected subscapularly with Freund adjuvant every 14 days into female New Zealand White rabbits. Antibody titers were monitored by immunoblotting with purified fusion protein as the antigen.

Immunoblot analysis. Proteins were separated on SDS–10% polyacrylamide gels and electrophoretically transferred to nitrocellulose paper (pore size, 0.2 μm; Schleicher & Schuell, Inc., Keene, N.H.) by using a Janssen semidry electroblotter. Blots were washed with RIPA buffer (0.15 M NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, 20 mM Tris hydrochloride [pH 7.4]) and blocked for 1 h at 37°C in RIPA buffer plus 1% gelatin (porcine skin; Sigma Chemical Co., St. Louis, Mo.) and 2% nonfat dry milk (Carnation). Blots were washed twice with RIPA buffer (10 min) and incubated for 1 h at 37°C with a 1/200 dilution (in RIPA buffer) of polyclonal rabbit antiserum raised against purified GalK–hPDE-1 fusion protein. After further washes, blots were incubated for 1 h at 37°C in RIPA buffer containing 2 μCi of ^{125}I-labeled protein A (1 mCi/ml; ICN Radiochemicals, Irvine, Calif.). Filters were washed extensively and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen at −70°C.

Compounds. Rolipram and SK&F94836 {2-cyano-1-methyl-3-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazine-3-yl) phenyl]guanidine} were synthesized at SmithKline Beecham Pharmaceuticals (by K. Erhard and W. Coates, respectively). RO 20-1724 was received as a generous gift from Hoffman-La Roche Inc., Nutley, N.J.

RESULTS AND DISCUSSION

Cloning and nucleotide sequence analysis of a human PDEase cDNA. A DNA fragment from the conserved region of a rat brain cDNA (RD1 [13]) encoding a low-K_m cAMP-specific PDEase, was used to probe a human granulocyte-macrophage colony-stimulating factor-stimulated monocyte cDNA library. Numerous overlapping clones were isolated, the longest (hPDE-1) containing a 2.6-kb insert. Sequence analysis of this clone revealed an open reading frame 2,055 bp in length, which predicts a protein of 686 amino acids with a molecular mass of ca. 77,000 daltons (Fig. 1). This size compares favorably with partially purified enzyme from human monocytes (J. White, personal communication). The open reading frame is flanked by 5′ and 3′ untranslated sequences of 265 and 295 bp, respectively. However, the clone does not contain either a polyadenylation signal or poly(A) tract, suggesting that the hPDE-1 cDNA is missing a portion of its 3′ untranslated region.

Identification of PDEase mRNA transcripts. Northern (RNA) blot analysis shows that hPDE-1 recognizes a moderately abundant 4.8-kilobase species, plus a minor species of lower molecular mass, present in primary human monocytes, human placenta, and U-937 cells (Fig. 2). Despite this potential heterogeneity, restriction analysis suggests that all of our positive clones (a total of 12) represent the same gene product (data not shown). These data suggest that only a single, low-K_m, cAMP-specific PDEase subtype may be expressed in monocytes. In addition, we have detected PDEase-specific mRNA species in all rat and mouse tissues surveyed by using our human cDNA clone as a probe (data not shown), an expected result given that our cDNA was isolated from a circulating cell type.

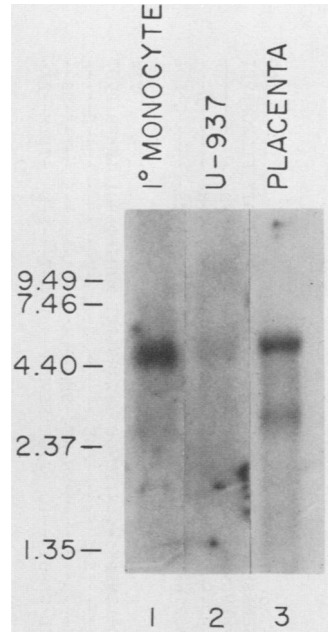


FIG. 2. Identification of hPDE-1 mRNA transcripts in human primary monocytes, human placenta, and U-937 cells. Northern blots were performed as described in Materials and Methods, with a ^{32}P-labeled fragment of hPDE-1, extending from the internal *Xho*I site to the 5′ polylinker *Xho*I site (Fig. 1), as a probe. Lanes: 1, 1 μg of poly(A)$^+$ RNA from primary monocytes; 2, 10 μg of polyA$^+$ RNA from U-937 cells; 3, 5 μg of poly(A)$^+$ RNA from placenta.

Analysis of the deduced hPDE-1 protein sequence. Alignment of the protein sequence predicted by hPDE-1 with the sequences of several other cloned PDEases, including RD1, DPD (also found to encode a low-K_m cAMP-specific PDEase) (10, 17), and *Drosophila dnc*$^+$ (7), reveals that they share a high degree of sequence similarity, particularly through two domains (Fig. 3): a short (40-amino-acid) region located amino terminally (hPDE-1 position 63), and a longer (ca. 270-amino-acid) region found in the central portion of the predicted sequence. Within this second region is found a perfectly conserved seven-amino-acid sequence (Glu-Leu-

FIG. 1. Restriction map, sequencing strategy, and nucleotide sequence of hPDE-1. (A) A partial restriction map of the hPDE-1 cDNA. Symbols: ■, coding region; ⟶, directions and approximate lengths of sequences obtained with oligonucleotide primers. Abbreviations: B, *Bgl*II; E, *Eco*RI; N, *Nco*I; Pv, *Pvu*II; S, *Sac*I; Sm, *Sma*I; X, *Xho*I. (B) Nucleotide sequence with the predicted amino acid sequence of the major open reading frame. Coordinates on the right indicate nucleotide and amino acid positions. Asterisks show the termination codons. The Met-48 residue (underlined) is the first possible translation start site in one of the COS-1 cell expression constructs (pRJB–hPDE-Met48) described in Materials and Methods. The boxed region represents a conserved sequence (see Fig. 3) also present in the cAMP-binding domain of the RII_a regulatory subunit of cAMP-dependent protein kinase (4, 30).

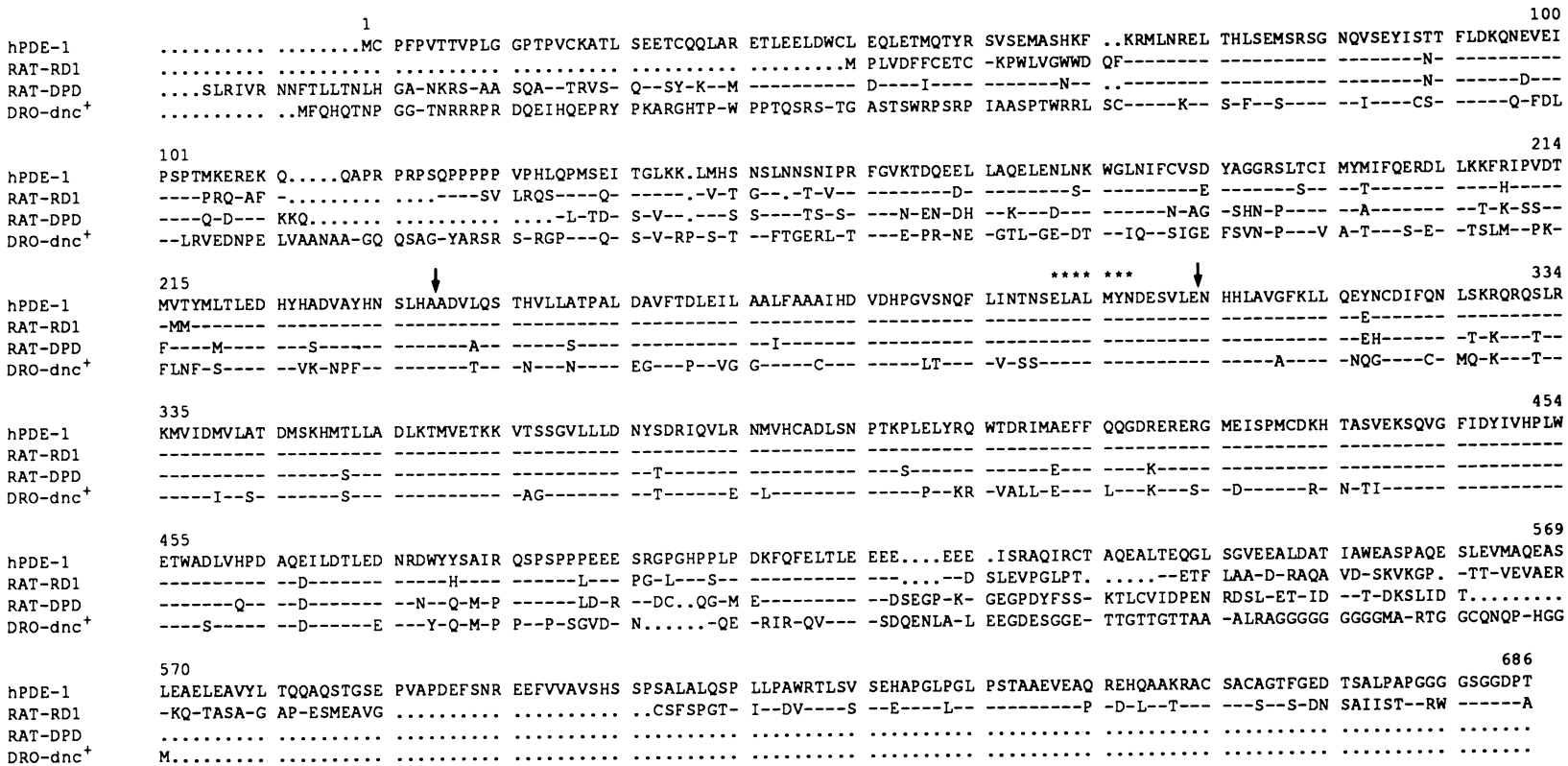


FIG. 3. Alignment of the deduced amino acid sequence of hPDE-1 with representative low-K_m cAMP-specific PDEases from other sources. RAT-RD1 and RAT-DPD represent distinct rat brain cAMP PDEases (10, 13); DRO-dnc$^+$ is *Drosophila* cAMP PDEase (7). Dashes indicate identical amino acid sequences; periods indicate sequence gaps. The conserved domains described in the text extend from hPDE-1 residues 63 to 104 and from residues 221 to 479. Asterisks represent a highly conserved sequence also found in the cAMP-binding domain of the RII_a regulatory subunit of cAMP-dependent protein kinase (4, 30). The amino acid sequence of the internal 189-bp *Pvu*II-*Xho*I fragment used as a probe in Fig. 6 is shown between the arrows.

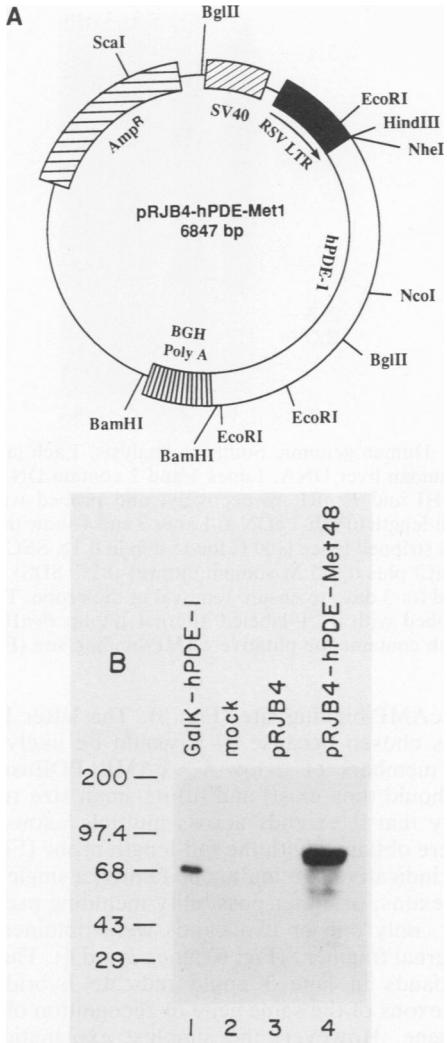


FIG. 4. Expression of hPDE-1 in COS-1 cells. (A) COS-1 cell expression vectors. pRJB4–hPDE-Met1 contains the entire coding sequence of hPDE-1 (▭) and was constructed as described in Materials and Methods. Expression is driven by the Rous sarcoma virus promoter (■). The plasmid also contains the bovine growth hormone transcriptional terminator (▥), simian virus 40 origin of replication (▨), and ampicillin resistance gene (▧). pRJB4–hPDE-Met48 (not shown) is analogous to pRJB4–hPDE-Met1, except that it lacks a small portion of the 5′ coding sequence and contains Met-48 as the first possible translation start site (Fig. 1). (B) Immunoblot analysis of hPDE-1 expression. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide), transferred to nitrocellulose, and probed with rabbit antiserum raised against purified GalK–hPDE-1 fusion protein produced in *E. coli* (see Materials and Methods). Lanes: 1, ca. 50 ng of purified GalK–hPDE-1 fusion protein; 2 to 4 ca. 5 μg of COS-1 cell lysate (lane 2, mock-transfected cells; lane 3, cells transfected with parent vector pRJB4; lane 4, cells transfected with pRJB4–hPDE-Met48).

Ala-Leu-Met-Tyr-Asn), which is also present in the cAMP-binding domain of the RII_a regulatory subunit of the cAMP-dependent protein kinase (4, 30). The sequences diverge substantially at the amino and carboxyl ends of the protein and appear completely unrelated within a 40- to 100-amino-acid block found just upstream of the carboxy terminus of the *Drosophila* PDEase. Of the 12 cysteine residues in the hPDE-1 protein sequence, 6 are conserved. Computer analysis of the hPDE-1 sequence predicts a protein with mixed

TABLE 1. Overexpression of cAMP PDEase activity in COS-1 cells[a]

Source	cAMP hydrolysis (nmol/min per mg of protein)	cGMP hydrolysis (nmol/min per mg of protein)
Mock	16.5	0.22
pRJB4	25.1	0.30
pRJB4–hPDE-Met1	178.0	0.52
pRJB4–hPDE-Met48	318.0	0.70

[a] Assays were conducted with 1 μM [^{3}H]cAMP or [^{3}H]cGMP as substrates (see Materials and Methods). Values represent the average of two separate determinations.

secondary structure and hydropathy characteristics; a search for internal homologies revealed no striking repeat patterns.

Expression of hPDE-1 in COS-1 cells. The above data suggest that hPDE-1 codes for a human cAMP-specific PDEase with a conserved internal domain that is likely to contain the active site for cAMP binding. To characterize the hPDE-1 gene product, we engineered the coding region for expression in COS-1 cells. Two COS-1 expression plasmids were constructed (pRJB4–hPDE-Met1 and pRJB4–hPDE-Met48) as described in Materials and Methods. pRJB4–hPDE-Met1 (Fig. 4A) contains the entire coding region of hPDE-1, beginning with the first ATG of the long open reading frame, whereas pRJB4–hPDE-Met48 is missing a small portion of the 5′ coding sequence and contains Met-48 as the first possible translation start site. COS-1 cells transfected with each of these plasmids expressed elevated levels of cAMP-specific PDEase activity (Table 1) (10- to 19-fold over background with mock-transfected cells). Expression was also monitored by immunoblotting (Fig. 4B). Total cell lysates of COS-1 cells transfected with each of these expression plasmids exhibited a major protein band of the appropriate size, which is recognized by antiserum raised against a GalK–hPDE-1 fusion protein produced in *E. coli* (see Materials and Methods) (Fig. 4B, lane 4; pRJB4–hPDE-Met1 not shown). Since no cross-reactive bands are detectable in mock-transfected cells or in cells transfected with pRJB4 alone, it appears that this antiserum does not cross-react with the endogenous PDEase responsible for the observed background cAMP-specific PDEase activity in COS-1 cells (Fig. 4B, lanes 2 and 3; Table 1).

The PDEase activity expressed in COS-1 cells was found to exhibit a high affinity for cAMP, with a K_m of 3.2 μM (Table 2). When assayed at substrate concentrations of 1 μM, cGMP hydrolysis was less than 1% of cAMP hydrolysis in the transfected-cell lysates (Table 1). Furthermore, the PDEase activity was not inhibited by cGMP (Table 2), eliminating the possibility that it corresponds to a low-K_m

TABLE 2. Apparent K_m and 50% inhibitory concentrations for the hPDE-1 recombinant gene product[a]

Source	K_m (μM)	IC_{50} (μM) of[b]:				
		Rolipram	RO 20-1724	SK&F94836	Zaprinast	cGMP
pRJB4–hPDE-Met1	3.2	0.9	5.3	>30	>30	>100
pRJB4–hPDE-Met48	ND[c]	1.0	5.8	>30	>30	>100

[a] Assays were conducted with 1 μM [^{3}H]cAMP as the substrate as described in Materials and Methods.
[b] IC_{50}, 50% inhibitory concentration.
[c] ND, Not done.

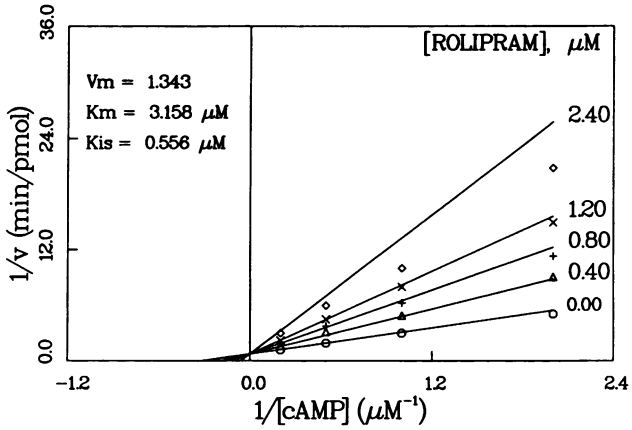


FIG. 5. Effect of rolipram on the hPDE-1 recombinant gene product expressed in COS-1 cells. The graph shows a representative double-reciprocal plot of cAMP activity in the presence of rolipram. The lines show results from nonlinear regression analysis of the original data fit to the equation for competitive inhibition. The apparent K_m and K_i values are indicated at the left. Velocities are expressed as picomoles of cAMP hydrolyzed per minute of incubation (using 0.13 μg of total cell protein from transfected cell lysates in a 0.1-ml reaction mix; see Materials and Methods).

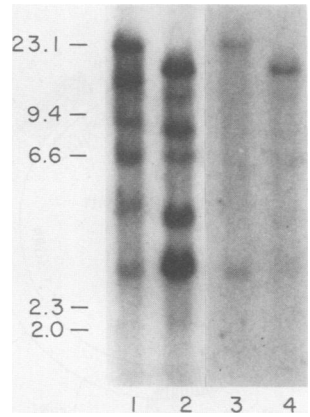


FIG. 6. Human genomic Southern analysis. Each lane contains 15 μg of human liver DNA. Lanes 1 and 2 contain DNA restricted with *Bam*HI and *Eco*RI, respectively, and probed with the ^{32}P-labeled full-length hPDE-1 cDNA. Lanes 3 and 4 show the same blot which was stripped twice (100°C for 15 min in 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% SDS) and autoradiographed for 3 days to ensure removal of the probe. The blot was then reprobed with a ^{32}P-labeled internal 189-bp *Pvu*II-*Xho*I fragment which contains the putative cAMP-binding site (Fig. 3).

cGMP-inhibited cAMP PDEase (3). The cAMP PDEase activity was competitively inhibited by rolipram, with a K_i of ca. 0.6 μM (Fig. 5) (50% inhibitory concentration, 0.9 μM [Table 2]). The enzyme activity was also inhibited by RO 20-1724 (Table 2). Inhibitors of the cGMP-specific PDEase (zaprinast) (34) and the cGMP-inhibited cAMP PDEase (compound SK&F94836) (35) had little or no effect on this PDEase activity (Table 2). From these biochemical characteristics, we conclude that the hPDE-1 recombinant gene product is a low-K_m, cAMP-specific, rolipram-sensitive PDEase. These observations are in good agreement with the substrate specificity, kinetics, and pharmacological sensitivity found for the rat counterparts, RD1 and DPD (17). The data also indicate that the 48 amino-terminal residues of the hPDE-1 gene product are not required for catalysis, since the truncated protein expressed by pRJB4–hPDE-Met48 shows a PDEase activity profile very similar to its full-length counterpart.

Low-K_m cAMP PDEases. (i) Potential isozyme heterogeneity. Protein sequences for several different classes of PDEases are now available, and comparisons indicate that they are members of a large, highly divergent multigene family. Our human clone is a low-K_m, cAMP-specific PDEase. It is apparent that in the rat the low-K_m cAMP-specific PDEases represent members of a moderately conserved multigene subfamily. For example, four rat clones have been reported (two from brain tissue, two others from testis tissue) (10, 13, 32), and sequence comparison shows that although all exhibit conservation through substantial portions of the protein, they are by no means identical, diverging dramatically elsewhere. Since the two recombinant rat enzymes that have been tested for activity appear very similar in terms of their substrate specificity, K_m, and selective inhibition by various compounds (10, 17), the functional significance of the different subtypes remains to be elucidated.

In an attempt to address the question of isozyme heterogeneity in humans, genomic Southern blots were probed with both our full-length cDNA and a short (189-bp) *Pvu*II-*Xho*I fragment from a highly conserved region containing the putative cAMP-binding site (Fig. 3). The latter DNA fragment was chosen because (i) it would be likely to detect multiple members of a low-K_m cAMP PDEase isozyme family (should they exist) and (ii) its small size reduces the possibility that it extends across multiple exons. Multiple bands were obtained with the full-length probe (Fig. 6, lanes 1 and 2), indicative of a multigene family, a single gene with multiple exons, or either possibility including pseudogenes. However, only one or two bands were obtained with the small internal fragment (Fig. 6, lanes 3 and 4). The presence of two bands in lane 3 could indicate hybridization to separate exons of the same gene or recognition of a second, related gene. However, the simplest explanation for the presence of a single band in lane 4 is that of one gene containing introns. In either case, these data show that the sequence contained in the internal fragment is not conserved across a family of PDEase genes. We conclude that, in contrast to the evidence for multiple genes in the rat (10, 13, 32), the human low-K_m cAMP PDEase cDNA described herein represents an isoform, derived from a single-copy gene, which may be related to, at most, one other gene.

(ii) Potential pharmacological significance. There is a growing body of biochemical evidence that the low-K_m cAMP PDEase(s) present in monocytes may act to degrade cAMP in airway smooth muscle (34) as well as in inflammatory cells (T. J. Torphy, G. P. Livi, J. M. Balcarek, J. R. White, F. H. Chilton, and B. J. Undem, Adv. Second Messenger Phosphoprotein Res., in press). Activation of the cAMP-cAMP-protein kinase cascade has been shown to mediate relaxation of airway smooth muscle (33, 35). This response can be mimicked by PDEase inhibitors which potentiate the biochemical and physiological responses to agents that stimulate adenylate cyclase. Therefore, selective inhibitors of the enzyme that we have described and begun to characterize may be useful in the therapy of asthma and certain types of inflammation.

It is interesting that whereas the genetic disruption of the *Drosophila dnc*$^+$ gene causes learning and memory dysfunction in flies, the pharmacological inhibition of the mammalian cAMP PDEases may affect mood. The antidepressant

properties of rolipram are thought to be mediated through specific inhibition of cAMP PDEase activity (29, 38). This suggests that the low-K_m cAMP PDEases, which function primarily to regulate cellular levels of cAMP, may also be intimately involved in the neurobiochemical processes that control information transfer in the brain. Thus, further studies of the recombinant human enzyme may be of great pharmacological significance in terms of our understanding of the mechanisms involved in the biochemical regulation of mood and human behavior.

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