Alternative Splicing of cAMP-specific Phosphodiesterase mRNA Transcripts

CHARACTERIZATION OF A NOVEL TISSUE-SPECIFIC ISOFORM, RNPDE4A8

Graeme B. Bolger‡§, Ian McPhee‡, and Miles D. Houslay†

From the Veterans Affairs Medical Center, 151M, and Huntsman Cancer Institute, Department of Oncologic Sciences and Department of Medicine (Hematology/Oncology), University of Utah Health Sciences Center, Salt Lake City, Utah 84148 and the Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom

In order to characterize the structure and regulation of members of the cAMP-specific phosphodiesterase (PDE) family (Type IV PDEs; PDE4 family), we have cloned from the rat a cDNA, pRPDE39, encoding a novel member of this family, which we call RNPDE4A8. Sequencing of the pRPDE39 cDNA shows it to be encoded by the rat PDE4A gene, but to differ from two other PDE4A transcripts, RD1 (pRPDE8; RNPDE4A1) and pRPDE6 (RNPDE4A5), by the presence of a unique region at its 5' end, consistent with alternative mRNA splicing. The pRPDE39 cDNA encodes a predicted protein of 763 amino acids, of which all but 21, located at the extreme amino terminus, are found in the pRPDE6 protein. Expression of pRPDE39 in COS cells produced a protein of 98 ± 1.4 kDa, as determined by immunoblotting with an antisera specific to the carboxyl-terminal regions of all PDE4A proteins, compared to a predicted value of 87.5 kDa. RNase protection analysis detected pRPDE39 mRNA only in testis. Immunoblotting of testis extracts demonstrated two bands of 97 ± 2 and 87 ± 3 kDa, the larger of which co-migrated with the band seen in COS cells expressing pRPDE39. COS cell expressed pRPDE39 partitioned between a high speed pellet (particulate) fraction (15% of protein; 8% of activity) and a cytosolic fraction. The particulate fraction had a K_m for cAMP of 3.3 ± 0.6 μM, and the cytosolic fraction a K_m of 5.4 ± 2.8 μM. The V_max values for the pRPDE39 protein, relative to the RD1 protein, were 0.16 ± 0.06 and 0.29 ± 0.05 for the particulate and cytosolic forms, respectively. The pRPDE39-encoded PDE activity could not be removed from the particulate fraction by high salt concentrations, or by nonionic detergents. The pRPDE39-encoded enzyme was inhibited by rolipram at an IC_50 of 0.5 ± 0.2 μM for the particulate form and 1.0 ± 0.2 μM for the cytosolic form, which are values typical of PDE4 family members. The highly tissue-specific distribution of the pRPDE39 mRNA suggests that the pRPDE39 protein functions to modulate a cAMP signaling pathway that is present largely, if not exclusively, in the testis.

The cAMP-specific phosphodiesterases (PDEs) are a large family of enzymes that are differentiated from other cyclic nucleotide PDEs by their high specificity and affinity for cAMP and by their inhibition by a specific class of compounds, including the antidepressant drug rolipram (1, 2). They are also the closest mammalian homologs of the dunce gene of Drosophila melanogaster, which was first isolated as a mutation affecting learning and memory (3). The cAMP-specific PDE proteins are encoded by four genes in mammals (PDE4A, PDE4B, PDE4C, and PDE4D), and at least three of these genes encode multiple PDE isoforms, generated from different alternatively spliced mRNA transcripts (reviewed in Refs. 2 and 4). The physiologic significance of this diversity of cAMP-specific PDE isoforms is not known. However, the rat PDE4 genes have different patterns of expression in tissues, suggesting, but not proving, that they have different functions (5-10). The various alternatively spliced mRNAs from each of at least three different PDE4 genes also have different regional patterns of expression in the brain (9). The proteins encoded by various alternatively spliced mRNAs from some of the mammalian PDE4 genes have, in some cases, been shown to have functional differences: for example, the different proteins encoded by various rat PDE4D mRNAs differ in their regulation by phosphorylation (10), and those encoded by different rat PDE4A transcripts differ in cellular localization (see below).

We and others have isolated cDNA clones derived from different mRNA transcripts of the rat PDE4A gene, studied the tissue distribution of these mRNAs, and analyzed the biochemical and pharmacologic properties and tissue localization of their encoded proteins (5, 6, 9, 11-15). The first rat PDE4A transcript to be described, RNPDE4A1, represented by the RD1 (5), and pRPDE8 (9) cDNA clones, encodes an enzyme of 610 amino acids that is expressed primarily in the brain, particularly the cerebellum (9, 12, 14). The RD1 protein is present exclusively in the high speed pellet, or particulate, fraction of cellular extracts, consistent with its associating directly or indirectly with membranes. A second rat PDE4A transcript, RNPDE4A5, represented by the pRPDE6 cDNA, is also expressed in brain and encodes a protein of 844 amino acids that is present in both particulate and cytosolic forms (9, 15). We now report a novel alternatively spliced mRNA transcript from the rat PDE4A locus, which we call RNPDE4A8 (using the accepted nomenclature (1)), and demonstrate that it is expressed almost exclusively in the testis. RNPDE4A8 encodes a rolipram-inhibited, cAMP-specific PDE of 763 amino acids that

* This work was supported by funds from the Huntsman Family Foundation (to G. B. B.) and the Medical Research Council, UK (to M. D. H.). DNA sequencing and oligonucleotide synthesis were conducted in core facilities of the Huntsman Cancer Institute, supported by Grant S-PO-CA42014 from NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L36467.

To whom correspondence should be addressed. Tel.: 801-582-1565 (Ext. 2155); Fax: 801-583-9624; E-mail: Graeme.Bolger@mcc.utah.edu.

1 The abbreviations used are: PDE(s), cyclic nucleotide phosphodiesterase(s); rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyridolidine-; UCR, upstream conserved region.
is present in both particulate and cytosolic forms. The highly specific tissue-specific distribution of the RNPD4A8 protein suggests that it functions in a signal transduction pathway that is present largely, or exclusively, in the testis.

**EXPERIMENTAL PROCEDURES**

Materials—A rat (Rattus norvegicus; Sprague-Dawley strain) brain cDNA library, cloned into the EcoRI site of Lambda ZAP, was obtained from Stratagene. DNA modifying and restriction enzymes were from New England Biolabs or Life Technologies, Inc. Culture media were from Life Technologies, Inc. Sequence reagents and radio-nucleotides and [3H]cAMP were from Amersham. Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates). Bradford reagent was from Bio-Rad. All other chemicals were from Sigma or Boehringer. Rolipram was a generous gift from Schering AG.

Isolation and Analysis of cDNA Clones—Procedures were as described by Sambrook et al. (16), unless otherwise specified. The rat brain cDNA library was screened with a hybridization probe from a portion of the rat pRPDE3 cDNA (rat PDE4D locus, corresponding to amino acids 83 to 333 of the alignment published previously (9)). Probes were labeled using random priming, and hybridization was done at 68°C in 6 × SSC, 0.5% SDS, and the final wash at 68°C in 0.1 × SSC, 0.1% SDS. cDNA clones were sequenced on both strands with Sequenase, or by dye-cycler sequencing, using an ABI prism sequencer (Perkin-Elmer). Amino acid sequence alignments were performed with the Genalign program (Intelectigenics).

RNase Protection Analysis—Total RNA was isolated from various tissues of rat (Rattus norvegicus) using the guanidine thiocyanate/CoCl<sub>2</sub> method (17). Single-stranded antisense RNA probes were generated from portions of cDNAs cloned under the control of the SP6 promoter (in pGEM3zf or pGEM4zf (Promega)). The probe for the 3′ terminus of pRPDE8 corresponded to nucleotides 2172 to 2433, outside of the coding region (9). The probes for the alternatively spliced 5′ portions of the cDNAs corresponded to amino acids 32 to 93 of pRPDE6 (Fig. 2) and to noncoding regions (9) of pRPDE8 (nucleotides 1 to 156) and pRPDE39 (nucleotides 1 to 205). RNase protection was done as described (18), with 10 μg of RNA per reaction. RNase digestion was done for 1 h at 35°C with RNase ONE (Promega), at a concentration of 3 units/reaction, with the conditions recommended by the manufacturer. Gels (6% acrylamide, 7 × urea, 1 × TBE) were autoradiographed at −70°C for 1-4 days with intensifying screens.

Generation and Expression of COS Cell Expression Vectors—Constructs containing the full ORF of RD1 (pRPDE8) cDNA in pSVL (Pharmacia Biotech Inc.) and pRPDE6 cDNA in pSVSPORT1 (Life Technologies, Inc.) have been previously described (12, 14, 15). In both of these constructs, the insert is expressed under the control of the SV40 early promoter. The met26RD1 construct expresses a truncated species of pRPDE8 (RD1) lacking the first 25 amino acids (12). To generate pCDNA-RPDE39, the complete ORF of pRPDE39 was cloned into pC DNA3 (Invitrogen), downstream of the cytomegalovirus promoter, using the BamHI and EcoRI restriction sites present in the cDNA. Transfection of monkey COS-7 cells was done as described previously (15).

Generation and Characterization of Antisera—A rabbit polyclonal antiserum, described previously (14), was generated against a synthetic peptide of 12 amino acids of the sequence COOH-TPGRWGSGGDPA-NH₂. This sequence corresponds to a portion of the carboxyl-terminal region in common with all of the known rat PDE4A proteins. Several antisera were generated independently in this manner, and all performed equally. The results in this paper were obtained with one antiserum (271), as described previously (14, 15).

Preparation and Fractionation of Tissue Homogenates—Homogenates from whole brain or testis were prepared as described previously for cerebellum (13, 14). These homogenates were then fractionated as follows. For the low speed pellet (P1), they were spun at 10,000 × g<sub>av</sub> for 10 min at 4°C. The supernatant from this step was then spun at 100,000 × g<sub>av</sub> for 1 h to yield a high speed pellet (P2; particulate) fraction and a supernatant (SN; cytosol) fraction.

Treatment of High Speed Pellet Fractions with Salt or Detergent—High speed pellet preparations (0.2 mg) were treated with KHEM buffer (50 mM KCl, 50 mM Hepes-KOH, 10 mM EGTA, 1.92 mM MgCl₂, pH 7.2) containing a range of NaCl or Triton X-100 concentrations, respectively. They were then incubated on ice for 30 min and then centrifuged at 100,000 × g<sub>av</sub> for 1 h at 4°C. The resulting pellet was resuspended in KHEM plus the appropriate NaCl or Triton X-100 concentration, and the pellet and supernatant were then analyzed by immunoblotting.

**RESULTS**

Isolation of cDNA Clones for the RNPD4A8 mRNA—To search for additional mRNA splice variants encoded by the rat PDE4A gene, a probe from the 5′ end of the pRPDE3 cDNA clone (rat PDE4D gene (9)) was used to screen a rat testis cDNA library. A testis library was chosen because of the work of Swinnen et al. (6) demonstrating that PDE4A transcripts were present in testis. Three different cDNA clones, all derived from the same mRNA, were each isolated multiple times. The longest of these, pRPDE39 (Fig. 1), contained one open reading frame (ORF), which was confirmed over its entire length by at least one other overlapping cDNA clone. pRPDE39 encodes a protein of 763 amino acids, of which all but 21, located at the extreme amino terminus, are contained in pRPDE6 (Fig. 2). The putative initiation ATG of the pRPDE39 protein is preceded upstream by termination codons in all three frames, although it only weakly satisfies the criteria of Kozak (25). Like pRPDE6, but unlike RD1 (pRPDE8), pRPDE39 contains complete upstream conserved regions 1 and 2 (UCR1 and UCR2).
UCR1 and UCR2 are regions of the amino-terminal sequence that are unique to the cAMP-specific PDEs and are highly conserved among species as evolutionarily divergent as *D. melanogaster* and humans (18). The point of divergence between pRPDE39 and pRPDE6 (indicated by A in Fig. 2) corresponds to the major point of alternative splicing in the *D. melanogaster* dunce mRNAs (3) and also in alternatively spliced mRNAs from the human PDE4A and PDE4D genes (18). This suggests that pRPDE39 and pRPDE6 are derived from alternatively spliced mRNAs, and that their unique 5' regions are encoded by separate exon(s).

**Tissue-specific Expression of the Alternatively Spliced Regions of the pRPDE6, pRPDE8, and pRPDE39 mRNAs—**

We wished to determine the contribution of pRPDE39 to the total amount of *PDE4A* mRNA in tissues. Transcripts from this gene had been shown previously to be expressed in a wide variety of tissues, although the relative contributions of the pRPDE6, RD1 (pRPDE8), and pRPDE39 transcripts in each of these tissues was not defined (5, 6, 10). However, we have reported previously the distribution of the pRPDE6 and RD1 (pRPDE8) mRNAs (9), and their respective proteins, in the brain (15). The distribution of the various alternatively spliced PDE4A mRNAs in various rat tissues was measured by RNase protection (Fig. 3). Single-stranded RNA probes complementary to the alternatively spliced regions of the pRPDE6, pRPDE8, and pRPDE39 cDNAs were used to probe total RNA isolated from various rat tissues. To determine the total amount of PDE4A mRNA in each of these tissues, a probe from the 3' terminus of pRPDE8, which detects all known PDE4A transcripts, was used as a comparison. Using this probe, transcripts from the PDE4A gene were found to be expressed in almost all tissues examined, as described previously (5, 6, 9, 10). This probe was designed to protect a 240-nucleotide fragment, but also generated a number of larger bands with the digestion and electrophoresis methods used (Fig. 3A). With a probe from the alternatively spliced region of the pRPDE8 cDNA, pRPDE8 transcripts were found to be most abundant in brain, with a roughly equal distribution in various regions of the brain, as reported previously (9). pRPDE6 transcripts were by far the most abundant in the olfactory bulb. The predominant band seen with the pRPDE6 probe was 135 nucleotides, which was smaller than the faint signals generated from nonolfactory brain tissues that we reported previously (9). A 135-nucleotide signal was also seen from cerebral cortex tissues on prolonged (3–4-day) exposures of the pRPDE6 autoradiogram. pRPDE39 transcripts were highly expressed in testis, with little or no expression in any other tissue examined.

**Expression of the pRPDE39 cDNA in COS Cells—**

To characterize the PDE encoded by the pRPDE39 cDNA, it was expressed in COS cells under the control of the cytomegalovirus intermediate early gene promoter. Immunoblotting of extracts from COS cells expressing pRPDE39, using an antibody raised against a dodcapeptide derived from the carboxyl-terminal region of the protein, demonstrated that the pRPDE39-encoded protein migrated as a single species of 105.5 kDa (Fig. 4), as compared to a predicted value of 87.5 kDa. By comparison, the RD1 protein and the pRPDE6 protein migrated at approximately 76 kDa and 107 kDa, respectively, as reported previously (15). All of these values are greater than those predicted on the basis of the predicted amino acid sequence of these proteins. It is possible that this may reflect particular conformational properties or the anomalous binding to SDS, of these proteins in SDS-polyacrylamide gel electrophoresis.

To determine whether a naturally occurring species of a...
molecular weight similar to the pRPDE39 protein is present in tissues, extracts from testis were analyzed by immunoblotting. Testis was chosen because our RNase protection experiments had demonstrated that pRPDE39 mRNA is present in testis. Immunoblotting of PDE4A species in testis, using our antibody to the carboxyl terminus, demonstrated the presence of a 97 ± 2-kDa and an 85 ± 3-kDa species (Fig. 4). The larger of these species co-migrated with COS cell expressed pRPDE39 (Fig. 4). By comparison, bands co-migrating with COS cell expressed RD1 and pRPDE6 proteins were seen in immunoblots of brain tissue (12, 15), where RD1 and pRPDE6 mRNAs are expressed

Fig. 3. Expression of the alternatively spliced mRNAs from the PDE4A gene in tissues. RNase protection assays with probes corresponding to the 3' terminus of pRPDE8 (A) and the alternatively spliced regions of pRPDE8 (B), pRPDE6 (C), and pRPDE39 (D) was performed using total RNA (10 μg) from various rat tissues. Arrowheads indicate the position of the RNA fragment protected by each probe. Size markers (in nucleotides) are indicated in the outside lanes, or as short horizontal lines on the sides of the panels, representing MspI-digested pBR322. Lanes are as follows: 1, probe without RNase; 2, kidney; 3, testis; 4, heart; 5, lung; 6, spleen; 7, liver; 8, skeletal muscle; 9, olfactory bulb; 10, brainstem; 11, cerebellum; 12, occipital cortex; 13, parietal cortex; 14, temporal cortex; 15, frontal cortex; 16, tRNA (10 μg).

Fig. 4. Expression of pRPDE39 protein in various tissues and in COS cells, as measured by immunoblotting. Immunoblots were performed with an antiserum specific for the carboxyl-terminal region of PDE4A proteins. A, extracts from testis tissue and COS cells expressing various plasmids. Lane 1, 150 μg of testis protein; lane 2, 50 μg of testis protein; lanes 3, 4, and 5, extracts from COS cells expressing the indicated plasmids (10 μg of protein). The positions of proteins used as size markers are indicated. All size determinations were done on a minimum of six independent determinations, using unstained molecular weight markers, as described previously (15). B, extracts from COS cells containing vector only (C) or vector expressing pRPDE39 (T). The immunoblots were done in the presence of various concentrations (μg/ml) of the peptide used to generate the antiserum. The single immunoreactive band found in the transfected cells and indicative of the pRPDE39 protein is marked with an arrow.

(9). We did not see bands co-migrating with the RD1 or pRPDE6 proteins in testis immunoblots, consistent with our RNase protection data (Fig. 3). The presence of an 85-kDa band in testis immunoblots was unexpected. This could reflect partial proteolysis of the 97-kDa band or, alternatively, a novel PDE4A protein, which could be encoded by a separate alternatively spliced mRNA.

Partitioning of pRPDE39 Protein between the Particulate Fraction and the Cytosol—We have determined previously that the RD1 protein is associated exclusively with the cellular particulate fraction (15), while the pRPDE6 protein is present in both particulate and cytosolic forms (15). We have also expressed in COS cells a truncated form of these proteins, called met26RD1 (Refs. 12 and 15). The met26RD1 protein is truncated at the amino terminus, beginning at the 26th codon of the naturally occurring RD1 protein (Fig. 2, indicated by B) and thus lacks any of the unique sequences encoded by the alternatively spliced regions of the naturally occurring PDE4A transcripts. The met26RD1 protein can be viewed as a "core" structure onto which the various PDE4A amino-terminal regions are "attached." The met26RD1 protein, as expressed in COS cells, is almost exclusively cytosolic, demonstrating that the amino-terminal regions of the RD1 and pRPDE6 proteins are necessary and sufficient for partitioning into the particulate fraction (15).
To determine the relative distribution of the pRPDE39 protein between the particulate fraction and cytosol, extracts of COS cells expressing pRPDE39 were fractionated into a low speed pellet (P1), a high speed pellet (P2), and cytosol, as described under "Experimental Procedures." The resulting fractions were then immunoblotted.

We have determined previously that the RD1 protein can be released from the particulate fraction by nonionic detergents, whereas the pRPDE6 protein cannot be released from this fraction by either detergents or by high salt concentrations (15). Therefore, we tested whether the pRPDE39 protein could be released from the particulate fraction by these treatments. COS cells expressing pRPDE39 were fractionated into high speed pellet (P2) and supernatant (cytosol) fractions. The high speed pellet (P2) fraction was treated with various concentrations of either Triton X-100 or NaCl, and the release of PDE protein from the particulate fraction was then analyzed by immunoblotting (Fig. 5). A single band of 97 ± 2 kDa was seen in homogenates and also in the high speed supernatant and high speed pellet (P2) fractions, with a trace amount in the low speed (P1) pellet. These data demonstrate that the pRPDE39 protein, when expressed in COS cells, partitions between both particulate and cytosolic fractions. Approximately 14.5 ± 2.5% of the PDE protein, as determined by immunoblotting, was present in the particulate fraction. In these respects, the pRPDE39 protein is similar to the pRPDE6 protein, but with one difference, that the pRPDE6 protein was found to be equally distributed between the high speed (P2) and the low speed pellet (P1) fractions (15).

A graph is shown in Fig. 6. Association of pRPDE39 enzyme with particulate fractions is unaffected by detergents or high salt concentrations. The high speed pellet (P2) fraction, which includes membranes, and a high speed superparticulate and cytosolic fractions. Approximately 14.5 ± 2.5% of the PDE protein, as determined by immunoblotting, was present in the particulate fraction. In these respects, the pRPDE39 protein is similar to the pRPDE6 protein, but with one difference, that the pRPDE6 protein was found to be equally distributed between the high speed (P2) and the low speed pellet (P1) fractions (15).

pRPDE39 Encodes a High Affinity cAMP PDE—The kinetic properties of the pRPDE39 enzyme were measured in homogenates of COS cells expressing pRPDE39. The average PDE activity in mock-transfected (vector only) COS cells was 34 ± 3 pmol of cAMP hydrolyzed/min/mg of protein (n = 6 separate transfections; mean ± S.D.). In pRPDE39-transfected cells, this rose to 452–832 pmol of cAMP hydrolyzed/min/mg of protein (range; n = 6). Double-reciprocal (Lineweaver-Burk) plots of cAMP substrate utilization showed apparent linearity over the substrate range examined (Fig. 7; Table I). The resulting fractions were then immunoblotted. The treatments were as follows: lane 1, 10% Triton X-100; lane 2, 1% Triton X-100; lane 3, 0.1% Triton X-100; lane 4, 5% Triton X-100, 2.5 mM NaCl; lane 5, 5 mM NaCl; lane 6, 0.5 mM NaCl; lane 7, 0.05 mM NaCl; lane 8, KH buffer as negative control.

The resulting fractions were then immunoblotted. The treatments were as follows: lane 1, 10% Triton X-100; lane 2, 1% Triton X-100; lane 3, 0.1% Triton X-100; lane 4, 5% Triton X-100, 2.5 mM NaCl; lane 5, 5 mM NaCl; lane 6, 0.5 mM NaCl; lane 7, 0.05 mM NaCl; lane 8, KH buffer as negative control.

A graph is shown in Fig. 7. The enzymatic activity of the particulate form is corrected for the gradient of cAMP substrate utilization. The cytotoxic form of the pRPDE39 enzyme is similar in activity to cytosol (IC50 = 0.5 ± 0.2 μM for the particulate form and 1.0 ± 0.2 μM for the cytotoxic form, respectively (Fig. 8). These values are typical for members of the PDE family. High concentrations of rolipram almost completely abolished detectable PDE activity in the extracts, consistent with the expressed enzyme being by far the major PDE activity in the transfected cells. The cytotoxic form of the pRPDE39 enzyme had an IC50 very similar to that of the cytotoxic form of the pRPDE6 enzyme and to that of the RD1 enzyme, which is present in the particulate fraction (Table I). The roughly 2-fold difference in IC50 between the particulate and cytotoxic forms of the pRPDE39 enzyme is different from that obtained previously for the pRPDE6 enzyme, where the particulate form was approximately 10-fold more sensitive to inhibition than the cytotoxic form (Table I).
been reported to date (5, 6, 9): RNPDE4A1, represented by the pRPDE39 protein, is a typical
mammalian cAMP-specific PDE (Refs. 9 and 18 and references therein), demonstrating that the pRPDE39 protein is a typical

presses in COS cells. A species of very similar size (97
6

PDE4A

with alternative splicing at its extreme 5
end. Compared with other PDE4A transcripts, the pRPDE39 mRNA has a unique pattern of expression, as it is found largely, if not exclusively, in testis. The pRPDE39 mRNA encodes a protein of 763 amino acids, which migrates as a 98 ± 1.4-kDa species when expressed in COS cells. A species of very similar size (97 ± 2 kDa) is seen on immunoblotting of testis extracts, suggesting that the 97-kDa testis band is encoded by the pRPDE39 mRNA. Like the pRPDE6 protein, but unlike the pRPDE8 (RD1) protein, the pRPDE39 protein is present in both cytosolic and particulate fractions. Neither high salt concentrations nor the nonionic detergent Triton X-100 could release the pRPDE39 protein from the particulate fraction. The Km for cAMP and IC50 for rolipram for enzymes encoded by RD1 (pRPDE6; RNPDE4A1), pRPDE6 (RNPDE4A5), and pRPDE39 (RNPDE4A8) were performed with 1 μM cAMP as substrate. Data are shown as mean ± S.D. for data obtained from 4 separate experiments, with PDE activity in the absence of inhibitor considered as 100%.

DISCUSSION

Many components of signal transduction pathways exist as multiple, closely related isoforms. In some cases, the different members of each of these families are encoded by separate genes. Additional diversity can be generated by other mechanisms, including alternative mRNA splicing, differential protein cleavage, and RNA editing. The functional consequences of this diversity are often unknown. In this study, we describe pRPDE39 (RNPDE4A8), which encodes a new CAMP-specific PDE isoform that differs from other proteins encoded by other rat PDE4A mRNAs. When compared to the structures of two other PDE4A mRNAs, pRPDE8 (RD1; RNPDE4A1) and pRPDE6 (RNPDE4A5), the pRPDE39 mRNA has a structure consistent with alternative splicing at its extreme 5‘ end. Compared with other PDE4A transcripts, the pRPDE39 mRNA has a unique pattern of expression, as it is found largely, if not exclusively, in testis. The pRPDE39 mRNA encodes a protein of 763 amino acids, which migrates as a 98 ± 1.4-kDa species when expressed in COS cells. A species of very similar size (97 ± 2 kDa) is seen on immunoblotting of testis extracts, suggesting that the 97-kDa testis band is encoded by the pRPDE39 mRNA. Like the pRPDE6 protein, but unlike the pRPDE8 (RD1) protein, the pRPDE39 protein is present in both cytosolic and particulate fractions. Neither high salt concentrations nor the nonionic detergent Triton X-100 could release the pRPDE39 protein from the particulate fraction. The Km for cAMP and IC50 for rolipram for the pRPDE39 protein are of the same order of magnitude as those of the proteins encoded by other rat PDE4A mRNAs (7, 8, 12–15, 26, 27) and also for other mammalian cAMP-specific PDEs (Refs. 9 and 18 and references therein), demonstrating that the pRPDE39 protein is a typical member of the cAMP-specific PDE4 family.

Five different cDNAs encoded by the rat PDE4A gene have been reported to date (5, 6, 9): RNPDE4A1, represented by the RD1 (5) and pRPDE8 (9) cDNAs; RNPDE4A2, represented by the RD2 cDNA (5); RNPDE4A3, represented by the RD3 cDNA (5); RNPDE4A5, represented by the pRPDE6 cDNA (9); and pRPDE39 (this article), which we tentatively call RNPDE4A8. The partial ratPDE2 cDNA clone (6) could belong to any of these groups, except RD2. We have discussed elsewhere why we believe that RD2 and RD3 most likely represent artifacts of cloning (2, 9). Therefore, the three cDNAs described in Fig. 1 represent all the known PDE4A mRNAs. However, other PDE4A cDNAs may be isolated in the future, as additional tissues are examined.

Our data demonstrate that there are multiple mechanisms for PDE4A regulation in the cell. First, there is regulation at the level of transcription or mRNA splicing, as the various alternatively spliced transcripts encoded by this locus are present in different levels in various tissues. We have previously shown this to be the case in the brain (9). Secondly, each of the proteins encoded by the various alternatively spliced transcripts may be differentially regulated in the cell. In this paper, we show that the cytosolic forms of both the pRPDE6 and pRPDE39 proteins have a roughly 2-fold difference in the Vmax values, as compared with that of their particulate forms. These differences, although small in magnitude, are reproducible and suggest that interaction with a membrane-associated component may be important in the regulation of PDE activity. Because the interaction of the pRPDE6 and pRPDE39 proteins with the particulate fraction could not be disrupted by either high salt, nor by nonionic detergents, it is likely that the proteins interact with a non-lipid moiety in this fraction, possibly a protein associated with detergent-insoluble cytoskeletal components. One potential problem with our data is that we have analyzed the biochemical properties of the pRPDE39 protein only by expressing it in COS cells. It is possible that the recombinant pRPDE39 protein may be processed differently in COS cells, as opposed to testis, or that overexpression of the protein in COS cells may affect its intracellular distribution. However, we consider that data obtained by expressing PDE cDNAs in COS cells is likely to provide a good representation of the native enzyme. This is based on our previous studies which yielded similar biochemical data for pRPDE6 and RD1 expressed in COS cells, compared to the corresponding native proteins in the brain (15).

Previously, we proposed that the different amino-terminal regions of the PDE4 proteins, like the amino-terminal regions of other PDEs, may regulate their biochemical properties (15). Our isolation of different members of the rat PDE4A family, all with identical catalytic regions, but differing in their amino-terminal regions, allows us to test this hypothesis. Within these amino-terminal regions are two novel and highly conserved regions of amino acid sequence, which we call upstream conserved regions 1 and 2 (UCR1 and UCR2; Ref. 18). UCR1 and UCR2 are conserved in organisms as evolutionarily distant as D. melanogaster and humans and appear to be distinct, as
they have no homology to each other, and are separated by a region of very low homology. We have now shown that the pRPDE6 and pRPDE39 proteins, both of which have extensive amino-terminal regions which include UCR1 and UCR2 (Fig. 1), have a $V_{\text{max}}$ that is 3- to 5-fold lower than that of the RD1 protein, which lacks UCR1 and the amino-terminal half of UCR2. This suggests that the amino-terminal regions of these proteins affect the conformation of the protein in a highly specific way, by attenuating the $V_{\text{max}}$ but not the $K_{\text{m}}$. These conformational and activity changes are a mechanism for regulating the PDE4A proteins.

One interesting property of the pRPDE39 mRNA is that it appears to be expressed largely or exclusively in testis. Partial cDNA clones derived from transcripts from all four PDE4 genes have been isolated from rat testis cDNA libraries (6–8, 10), and complete cDNAs encoded by two of these genes, PDE4B and PDE4D, have been isolated from these libraries (7, 8). A 67-kDa protein which reacts with antisera specific to PDE4D proteins has been purified from rat testis and is probably encoded by the RNPD4D1 mRNA (28). However, all of the rat PDE4B and PDE4D cDNAs that have been isolated from testis have also been detected in tissues outside the testis, including the brain (9). A complete cDNA from the rat PDE4C gene has not been reported, but PDE4C transcripts are expressed in a number of tissues (6, 9). pRPDE39 is the first complete PDE4A transcript to be isolated from testis. Our RNAse protection analysis and immunoblotting of extracts from testis are consistent with pRPDE39 being a major PDE4A-encoded protein in testis.

The physiologic role of the pRPDE39 protein is not known. However, it may play a role in a cAMP signaling pathway that is largely, or exclusively, present in testis. In males, the gonadotrophic peptide hormones follicle-stimulating hormone and luteinizing hormone act almost exclusively on the testis, binding to serpentine seven-transmembrane G-protein-coupled receptors. Activation of these receptors increases adenylyl cyclase activity (29–31). The pRPDE39 protein, as well as other cyclic nucleotide PDEs in the testis, may modulate the actions of these hormones, as suggested previously by other groups (7, 8, 27, 32, 33).

Acknowledgments—We thank R. J ones and M. Robertson for technical assistance.

---

2 G. B. Bolger, unpublished data.