We report the cloning, expression, and characterization of a new family of cyclic nucleotide phosphodiesterase (PDE) that has unique kinetic and inhibitor specificities. A clone corresponding to the C terminus of this PDE was initially identified by a bioinformatic approach and used to isolate a cDNA that is likely full-length. This novel PDE, designated as MMPDE9A1, shows highest mRNA expression in kidney with lower levels in liver, lung, and brain. The mRNA size by Northern blot analysis is approximately 2.0 kilobases, and the cDNA encoding PDE9A1 is 1929 base pairs in length. The largest open reading frame predicts a protein of 534 amino acids with a molecular mass of 62,000 Da. When expressed in COS-7 cells, PDE9A1 activity was not inhibited well by either the nonselective inhibitor 3-isobutyl-1-methyl-xanthine or the new selective PDE5 inhibitor, sildenafil, but it is inhibited by the PDE1/5 inhibitor (+)-cis-5,6a,7,8,9 hyyl phenylmethyl-5-methyl-cylopent(4,5]imidao[2,1-b]purin-49(3H)one (SCH51866) with an IC_{50} of 1.55 \mu M. This new phosphodiesterase is highly specific for cGMP. Its K_{m} of ~0.07 \mu M for cGMP is the lowest yet reported for a PDE, being at least 40–170 times lower than that of PDE5 and PDE6, respectively.

The cyclic nucleotides cAMP and cGMP serve as second messengers for a wide variety of extracellular signals such as neurotransmitters, hormones, light, and odors. The diverse cellular and behavioral responses to these second messengers are mediated by the action of cAMP and cGMP on their intracellular targets, which include kinases, ion channels, transcriptional activators, and several isoforms of phosphodiesterases (PDEs).\(^1\) Accordingly, these responses are regulated by the rates of synthesis of cyclic nucleotides by cyclases and their degradation by PDEs to biologically inactive 5’ monophosphate nucleosides.

Seven\(^2\) different gene families of PDEs previously have been isolated based on their distinct kinetic and substrate characteristics, inhibitor profiles, allosteric activators and inhibitors, and amino acid sequence (1). Family 1 is activated by Ca^{2+}/calmodulin and hydrolyzes both cAMP and cGMP; family 2 is stimulated by cGMP, and both cAMP and cGMP serve as substrate; family 3 is distinguished by cAMP hydrolysis that is inhibited by cGMP; family 4 is cAMP-specific; family 5 binds cGMP at a noncatalytic site and specifically hydrolyzes cGMP; family 6 is the retinal PDE that is inhibited by a γ subunit in the absence of activated transducin and hydrolyzes cGMP; and family 7 is a very low K_{m} cAMP-specific PDE. Not only does each family of PDE have specialized substrate and regulatory features, but each PDE family and even members within a family also exhibit tissue-, cell-, and subcell-specific expression patterns and therefore participate in distinct signal transduction pathways. The precise cellular and subcellular profile of PDE expression then will determine the cyclic nucleotide phenotype of a cell and how it responds to first messengers. Identifying and characterizing these functionally distinct PDEs is therefore crucial for our understanding of the mechanisms by which cyclic nucleotides moderate their biologic effects.

We report here the cloning, expression, and characterization of a previously unknown PDE designated as MMPDE9A1 (2). This PDE represents a new gene family because it shares less than 50% amino acid identity in the conserved catalytic domain with the other seven PDE families. A search of GenBank\(^*\) reveals that PDE9A1 has slightly higher sequence homology to a recently described Dictyostelium discoideum PDE referred to as Rega (3) rather than other mammalian PDEs. Additionally, expression of PDE9A1 in COS cells results in functional PDE activity that is unique kinetically from the other seven families in that it is cGMP-specific and has the lowest K_{m} for cGMP reported so far for a PDE.

**MATERIALS AND METHODS**

**Data Base Searching for EST PDE Sequences**—The amino acid sequence of MMPDE1A2 was used as a query to search the data base of expressed sequence tags (4, 5). The program used was the Basic Local Alignment Search Tool (BLAST) (6) accessed from the data base search and analysis Search Launcher (7). This search resulted in many EST sequences with homology to the PDE1A2 query. Each of these EST sequences were then used as queries in a BLASTN search of GenBank\(^*\) to determine whether they represented different but known PDEs or whether the EST sequence represented a truly unknown PDE. EST clone identification number 404030 was isolated in this manner as a sequence that appeared to represent a novel PDE.

**Sources of EST Clones**—Clone 404030 was ordered from the American Type Culture Collection. Clones 420451 was ordered from Genome Systems, Inc.

**DNA Sequencing and Sequence Assembly**—Plasmid DNA was prepared using the SNAP kit (Invitrogen). Primers were designed using the program Amplify (freeware by William Engels, Genetics Department, University of Wisconsin, Madison, WI). Sequencing was done using ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer), and sequencing reactions were purified using Centri-Sep columns (Princeton Separations). Sequences were assembled using the program Sequencer 3.0 (Gene Codes Corporation).

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\(^1\) The abbreviations used are: PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methyl-xanthine; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; kb, kilobase(s); SCH 51866, (+)-cis-5,6a,7,8,9 hyyl phenylmethyl-5-methyl-cylopent(4,5]imidao[2,1-b]purin-49(3H)one; ANP, atrial natriuretic peptide; PCR, polymerase chain reaction; MOPS, 3-[N-morpholino]propanesulfonic acid.

\(^2\) Note that an eighth family of cyclic nucleotide phosphodiesterases has also been identified by our lab and by Fisher et al. Fisher, D. A., Smith, J. F., Pillar, J. S., St. Denia, S. H., and Cheng, J. B. (1998) Biochem. Biophys. Res. Commun. 246, in press.

Identification and Characterization of a Novel Family of Cyclic Nucleotide Phosphodiesterases*

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Primers Used—The primers used were: Krace.S1, ggatagggggcggctggcgaactagct; 4040AS2, ggctccacatggaagtcagcacaac; PDE9stp.AS2, ccttccatacactggtagctaaactggtgtaag; PDE9estp.AS2, ctggagaagtggatgctgtggctgctgc; Gal4ActAS.pri, gaattctgagttgccagcagccgacag; AP1, ccaacctatactgaaagtctggcagggc; AP2, actcaatagctggactggcaggg.

DNA Probe Synthesis and Northern Blotting—DNA probes were generated from EST clone 404030 and B-actin using Prime-It RmT random primer labeling kit (Stratagene). 32P-PdCTP at 600 Ci/mmol was used, and the reaction product was purified using Centri-prep columns. Multiple tissue mRNA blot was purchased from CLONTECH. Prehybridization, hybridization, and washing was done according to the manufacturer’s guidelines. Hybridization was done using 2 × 106 cpm/ml in a final volume of 20 ml at 42 °C overnight.

Library Screening—EST clone 404030 was used to screen approximately 700,000 plaques from a mouse kidney cDNA library (CLONTECH). Briefly, the library was incubated with an overnight culture of €600H1 Escherichia coli and plated onto NZY plates with NZY plus top agarose and incubated for 8 hr. Plates were then stored overnight at 4 °C. Nitrocellulose filters were placed on top of these plates for 5 min in duplicate and denatured for 5 min in 1.5 M NaCl and 0.5 M TTX (supplied with Advantage polymerase mix) at 65 °C. Filters were then washed in 2 × SSC and incubated in hybridization solution (50 ml Tris-HCl, 1% SDS, 10% dextran sulfate, 1 M NaCl) for 1 h at 65 °C. 5 × 106 cpm/ml of boiled probe was added, and hybridization was continued overnight. Filters were then washed in 2 × SSPE for 15 min twice at room temperature and then twice in 0.1 × SSPE, 0.5% SDS for 30 min at 65 °C. Filters were then exposed to autoradiographic film overnight, and positive plaques were picked and stored in 50 ml Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgSO4, 2% gelatin until further rounds of screening were done. This resulted in two independent clones, clones 2.1 and 6.1, which were identical and contained the sequence of EST clone 404030 in the correct orientation. The cDNA as reported here has been confirmed to exist as a single mRNA species by PCR of kidney cDNA using primers Krace.S1 and PDE9.estp.AS1 (Fig. 1). Expression of PDE9A1—PDE9A1 cDNA subcloned in PCR3.1 was purified using Qiagen tip-500 columns. 10 μg of DNA was electrophorized into 2 × 105 COS-7 cells/plate in a 0.4-cm electrophoresis cuvette at 250 microfarad and infinite resistance using 300 V, 50 watts, and 50 mA. The amount of PCDNA3.1 vector (Hygro) alone was also transfected under the same conditions as a negative control for expression. Four plates for each construct were transfected and plated onto 100-mm dishes and kept in 5% CO2 for 24 h. After this period fresh medium was added, and cells were placed in 10% CO2 for another 24–48 h. Each set of four plates was pooled and harvested in 4 ml of homogenization buffer containing 40 mM Tris-HCl, pH 7.5; 15 mM benzamidine; 15 mM mercaptoethanol; 1 μg/ml pepstatin A; 1 μg/ml leupeptin; and 5 mM EDTA. This was homogenized in a Dounce homogenizer on ice with 25 strokes, and then 1 volume of glycerol was added. This preparation was stored at −70 °C in aliquots and did not lose appreciable activity over 2 months.

Kinetics and Inhibitor Studies—All PDE assays were done according to the method of Hanssen and Beavo (8) in a buffer containing 40 mM MOPS, pH 7.5, 0.8 mM EGTA, 15.0 mM magnesium acetate, 0.2 mg/ml bovine serum albumin, and (3H)GMP (25,000 cpm/reaction) in a final volume of 250 μl. All assays were done in triplicate, and reaction times and enzyme amounts were kept such that the lowest substrate concentration gave no more than 30% hydrolysis. PDE9 activity is defined as the total specific activity in PDE9-transfected COS cells minus the background specific activity of mock transfected COS cells. cGMP hydrolytic activity was approximately 8–16-fold higher in PDE9-transfected cells compared with mock transfected cells at 1 μM cGMP and approximately 50-fold higher at 0.06–0.1 μM cGMP. Thus the basal cGMP hydrolytic activity in COS-7 cells was not a significant contributor to the total activity in PDE9-transfected cells. Inhibitor studies were done without added unlabeled cGMP to keep the final substrate concentration low so that IC50 values would approximate the K1 value. IBMX, erythro-9-(2-hydroxy-3-nonyl)-adenine, and dipyridamole were obtained from Sigma. Zaprinast was a gift from May & Baker LTD, UK. Rolipram was obtained from BioMol (Plymouth Meeting, PA). SCH 51866 was a gift from Schering-Plow Research Institute, and sildenafil was a gift from Pfizer Central Research (Sandwich, UK). Enoximone was a gift from the Merrell Dow Research Institute.

RESULTS AND DISCUSSION

Cloning, Tissue Distribution, and Predicted Sequence of PDE9A1—We have cloned and characterized a cDNA encoding a new cyclic nucleotide phosphodiesterase that represents a previously unknown gene family. A partial cDNA representing this novel PDE, which had low but significant homology to the C terminus of calmodulin PDE MMPDE1A2, was initially isolated from the data base of expressed sequence tags. This sequence (mouse embryo EST clone 404030) did not match any GenBank® PDE sequence with high homology but did match PDEs in general better than other GenBank® sequences, suggesting that this sequence may represent a PDE of a new family. This EST was used to probe a mouse multiple tissue Northern blot to assess tissue distribution and mRNA size. (Fig. 2) Northern blot analysis indicates that mRNA represent-
because no start site with a Kozak consensus (10) was present. a cDNA that still appeared to be truncated at the 5’ end of our resulting cDNA. Because the mRNA size by Northern blot analysis was 2 kb, Northern blot analysis indicates that PDE9 is expressed at highest levels in kidney, followed by liver, brain, and lung.

**Fig. 2.** Northern blot analysis of PDE9 expression. The Northern blot contains 2 µg of mouse poly(A) RNA per lane. The blot was hybridized with either PDE9 or B-actin probes as described under “Materials and Methods.” Relative size (kb) is indicated on the left based on mobility of the RNA ladder. B-actin was measured to ensure roughly equal loading of mRNA on the blot. This B-actin probe cross-hybridizes to shorter forms of actin (α and γ), particularly in skeletal muscle and heart. B-actin is a 2-kb mRNA, and alignment of this signal with the Northern blot using the EST probe demonstrated that these two mRNAs co-migrate, allowing a good estimation of the EST mRNA size to also be 2 kb. Northern blot analysis indicates that PDE9 has a high level of expression in mouse kidney and lower levels of expression in liver, lung, and brain. This mRNA is estimated to be 2.0 kb in size.

Based on this result we set forth to clone the full-length cDNA for this EST from appropriate tissues to determine whether it did indeed represent a novel PDE. Several strategies for cloning the full-length cDNA of this EST were used, including hybridization screening of λ phage library, PCR screening of plasmid cDNA library, 5’ RACE, and EST data base walking, which involves using additional sequence information to screen EST data bases for longer EST clones overlapping the original truncated clones (Fig. 1). DNA probes were prepared from the 404030 EST insert fragment and used to screen roughly 700,000 mouse kidney AgT10 plaques. The resulting plaque pure clones were PCR amplified from λ phage and subcloned into PCR2.1 for sequencing. PCR analysis indicated clones 2.1 and 6.1 were both approximately 1 kb in size. Because the mRNA size by Northern blot analysis was 2 kb, this suggested that these clones were truncated. Sequencing of these clones demonstrated this to be the case. Clones 2.1 and 6.1 are identical and contain the sequence of EST clone 404030 to a polyadenine sequence. Simultaneous to the hybridization library screen we also designed primers complementary to the EST cDNA sequence to screen additional libraries by PCR for sequence 5’ to the EST sequence. This sequence was necessary for determining whether the EST actually encoded for a PDE because the catalytic domain sequence was predicted to lie 5’ to the sequence of 404030. Southern blot analysis of this PCR reaction demonstrated two bands that were specific to our EST sequence. Subsequent nested PCR reactions were done that resulted in a 1.6-kb cDNA that was subcloned into PCR2.1 TA vector for sequencing.

This insert was sequenced and contained not only the sequence of EST clone 404030 but also the sequence that appeared to encode for a PDE catalytic domain containing the PDEase signature motif of HDXXHYN (9). Additionally, this PDE appeared to belong to a novel family of cyclic nucleotide phosphodiesterases in that the predicted conserved domain shared less than 50% sequence identity with the conserved domains of members of the other seven known families (Fig. 3).

**Fig. 3.** PDE9 represents a novel family of phosphodiesterase. This figure represents the results of alignment analysis comparing the catalytic domain of PDE9 (defined by the boxed amino acid sequence in Fig. 4A) to the catalytic domains of representatives of seven other PDE families. Typically phosphodiesterases have 80 to >90% amino acid sequence identity within their catalytic domains when compared within a family. PDE9 has less than 50% sequence identity within this domain, confirming that this PDE represents a new phosphodiesterase gene family.

Walking the EST data base was done by using the additional rat brain cDNA sequence as a query to screen the data base using BlastN to find additional EST clones that contained the new 5’ end sequence and may also contain an additional sequence not cloned from the PCR screen of the rat brain cDNA library. This EST walking resulted in the isolation of mouse embryo EST clone identification number 420451, which contained an additional 18 bases 5’ to the rat brain cDNA sequence. These additional 18 bases contained an in-frame start methionine with a very strong Kozak consensus sequence (10). This clone also contains the sequence of EST clone 404030 and is truncated at the 3’ end internal NotI site consistent with the use of NotI in the generation of this library. 5’ RACE of mouse kidney and day 17 embryo cDNA was also done, and sequencing of these cDNAs cloned into PCR2.1 yielded no additional sequence but confirmed the 5’ end of our resulting cDNA.

The subsequent cDNA compiled from the overlapping EST clone 420451 and clones 2.1 and 6.1 of the mouse kidney library screen generate a sequence that is 1929 base pairs in length. PCR of mouse kidney cDNA using primers Krace.S1 and PDE.stp.AS1 and sequencing of this clone demonstrates that the resultant overlapping cDNA clone presented here exists as a single mRNA species (Fig. 1). It appears unlikely that this cDNA is not full-length because no additional 5’ sequence was found in either the EST data base or by 5’ RACE of both mouse kidney and mouse embryonic cDNA. Furthermore, the cDNA size is in good agreement with the corresponding 2.0-kb mRNA size observed by Northern blotting, and the start methionine follows the rule of Kozak (10). A human isoform of this family (HSPDE9A2), which shares 93% sequence identity to MMPDE9A1, has been recently identified (11). This clone contains a putative human methionine identical to the putative start methionine found in MMPDE9A1, and the predicted amino acid sequence above this start methionine is not conserved between these two isoforms, arguing that this region is untranslated. Additional methionines or in-frame stop codons have been found upstream of the putative start methionine in either the mouse or human cDNAs. Because the cDNA is slightly smaller than the mRNA and because no in-frame stop codons have been found, until either the amino acid sequence of...
the endogenous PDE9 or an in-frame stop codon in the putative 5'-untranslated region is discovered, the assignment of the start methionine presented here will remain tentative despite the above evidence, indicating this sequence is likely to be full-length. Although the mouse and human PDEs are certainly of the same family, with identical kinetics and inhibitor profiles, they are different in that MMPDE9A1 does not contain an insertion of 60 amino acids found within the region N-terminal to the conserved catalytic domain of HSPDE9A2. These two PDEs may represent distinct splice variants of the same family, or this variance may be attributable to a species difference. The fact that PDE9 is conserved from mouse to humans argues that this PDE family is functionally significant to these organisms. The predicted protein sequence of MMPDE9A1 is 534 amino acids in length with a predicted molecular mass of 62 kDa. (Fig. 4A) This start methionine and the resultant open reading frame is functional when expressed in COS-7 cells (see below).

Comparison of the PDE9A1 Conserved Domain to Other Phosphodiesterases—All mammalian PDEs characterized to date contain a conserved region of approximately 250 amino acid residues representing the catalytic domain of each of these enzymes (12). Many residues within this region are either absolutely or chemically preserved across species, from yeast to humans, and between PDE families. PDE9 is interesting in that three of these very highly conserved amino acids are divergent and all three cluster to the C-terminal end of the catalytic domain. The divergent amino acids identified here in mouse PDE9 are conserved in human PDE9 (11) also. Fig. 4B is an alignment of the C-terminal end of the catalytic domain from representatives of each of the eight families of PDEs. The first divergent substitution (1, Fig. 4B) is a conservative switch from a phenylalanine that is absolutely preserved among all other known PDEs to a tyrosine in PDE9. RegA does not contain this amino acid change. Position 2 shows the switch from a glycine to a serine found in both PDE9 and RegA. Position 3 represents a switch from the chemically conserved isoleucine, leucine, valine residues found in families 1–7 to a lysine in PDE9. Interestingly RegA is similar to PDE9 here also, containing an arginine at this position. Accession numbers for sequences aligned in Figs. 3 and 4B are as follows: PDE5, L16545; PDE6, X55968; PDE2, U21101; PDE1, L01695; PDE4, U09457; PDE7, U68171; PDE9, J22867; RegA, U60170.

**FIG. 4.** **Sequence of PDE9A1 and unique features of the catalytic domain.** The cDNA for PDE9A1 is 1929 base pairs in length and is predicted to encode a 534-amino acid enzyme with a predicted molecular mass of 62,000 Da. A, untranslated DNA sequence and amino acid sequence of PDE9A1. Above the 5'-untranslated DNA sequence is an alignment of the Kozak consensus sequence, demonstrating the strong Kozak consensus at the start methionine for PDE9. The boxed amino acid domain represents the catalytic domain based on conservation of this region to other phosphodiesterases. The start codon and stop codon in the DNA sequence are underlined. B, unique features of PDE9 conserved domain. Amino acid alignment of representatives of the seven other PDE families to PDE9 and RegA. Open boxes represent either chemically conserved residues or residues conserved among all known mammalian PDEs. 1, 2, and 3 mark where PDE9 diverges from all previously known PDEs. Position 1 is a conservative change from a phenylalanine to a tyrosine in PDE9. RegA does not contain this amino acid change. Position 2 shows the switch from a glycine to a serine found in both PDE9 and RegA. Position 3 represents a switch from the chemically conserved isoleucine, leucine, valine residues found in families 1–7 to a lysine in PDE9. Interestingly RegA is similar to PDE9 here also, containing an arginine at this position. Accession numbers for sequences aligned in Figs. 3 and 4B are as follows: PDE5, L16545; PDE6, X55968; PDE2, U21101; PDE1, L01695; PDE4, U09457; PDE7, U68171; PDE9, J22867; RegA, U60170.
reading frame reported here into the vector PCR3.1 for expression in mammalian cells. This construct was transfected into COS-7 cells, and PDE activity was assayed after 48–72 h. A low $K_m$ cGMP PDE activity that was significantly higher (6–18-fold higher, depending on transfection batch) in PDE9-transfected cells compared with PCDNA-3 alone-transfected cells was detected. This PDE9 activity does not appear to hydrolyze cAMP, and cGMP activity is not appreciably effected by cAMP, up to 100 μM (data not shown). This indicates that PDE9 is very specific for the hydrolysis of cGMP alone. Kinetic assays were done in triplicate on two different batches of COS-7 cell homogenates transfected with PDE9. Endogenous cGMP hydrolysis from vector only-transfected cells were subtracted from corresponding assays of PDE9-transfected cells. These assays demonstrate that this PDE has a $K_m$ of 0.07 μM ± 0.03 (average of five separate assays) (Fig. 5). This is the lowest $K_m$ for cGMP observed for a phosphodiesterase to date.

Because PDE9 is specific for the hydrolysis of cGMP, it is of interest to compare this PDE with other cGMP specific PDEs. Although a previous report (13) has speculated on the existence of novel cGMP phosphodiesterases based on kinetic and inhibitor profiles of a partially purified activity, this activity is not obviously related to any of the known mammalian PDEs specific for the hydrolysis of cGMP, PDE5 and PDE6. PDE6, also known as the retinal PDE, is tightly regulated by the binding of an inhibitory γ subunit. γ-mediated inhibition of PDE activity is relieved by activated transducin in response to light. PDE activation is also thought to be regulated by the binding of cGMP at noncatalytic sites, although this mode of regulation of PDE6 is not well understood (14–17). PDE6 has a $K_m$ of 17 μM (18), approximately 170 times higher than that of PDE9. PDE5 is also cGMP-specific and binds cGMP at a noncatalytic cGMP-binding site. Binding of cGMP at this noncatalytic site is expected to confer some type of regulatory effects on PDE activity, although no direct effect has been demonstrated to date (1). This PDE has a $K_m$ of 4 μM (19), which is at least 40 times higher than that of PDE9. PDE9 is the first cGMP-specific PDE to be identified that does not contain a region homologous to that of the noncatalytic cGMP-binding sites conserved between both PDE5 and PDE6 (20).

**Inhibitor Profile of PDE9A1—** Inhibitor studies were performed on COS-7 cell homogenates expressing PDE9 activity. PDE9 activity varied between transfections from 6- to 18-fold higher than COS-7 cGMP PDE endogenous activity. Any background endogenous cGMP PDE activity was subtracted, and inhibitor vehicle effects were likewise subtracted from the effects of inhibitor. These assays were done in triplicate on two separate transfection batches. No unlabeled cGMP was added to these reactions so that the final substrate concentrations were determined only by the addition of tracer [3H]cGMP to the reaction (11.3 nM). At these concentrations the $IC_{50}$ should approach the $K_m$. Although PDE9 demonstrates cGMP-specific PDE activity, this PDE is not inhibited well by most of the PDE inhibitors, including the nonspecific inhibitor IBMX ($IC_{50}$ greater than 200 μM) (Table I). It is important to note that this PDE is also not inhibited by the PDE5-specific inhibitor sildenafil (21) nor the PDE5 and PDE6 inhibitor dipyridamole at doses within ranges considered specific. Zarinprast, another PDE5 and PDE6 inhibitor, will inhibit PDE9 at moderately high concentrations (29 μM). Interestingly, although PDE9 is not inhibited well by IBMX or the PDE5 or PDE6 inhibitors, it is inhibited by the relatively new PDE 1 and PDE5 inhibitor SCH 51866 with an $IC_{50}$ of 1.55 μM. This is only 15 times higher than the $IC_{50}$ of SCH 51866 for both PDE1 and PDE5, both of which have an $IC_{50}$ of approximately 0.1 μM (22).

Few studies examining the biologic effects of SCH 51866 have been done to date; however, one such study has found this drug to have antiplatelet, antiproliferative, and hemodynamic effects (22). In this study, SCH 51866 inhibited platelet aggregation with an IC50 of 10 μM. Additionally, this compound also inhibited platelet adhesion, and platelet accumulation of cGMP was noted at these inhibitor concentrations. In balloon angio-plasty injury of rat carotid arteries, SCH 51866 inhibited neointima formation. Furthermore, SCH 51866 dose-dependently reduced blood pressure in spontaneously hypertensive rats. It is possible that some of these results may be attributable to inhibition of PDE9 in addition to inhibition of either PDE1 or 5. Inhibition of PDE9 by SCH 51866 may also point to possible starting compounds for future studies assaying for more selective and potent inhibitors of PDE9. SCH 51866 may be useful in future studies of PDE9 function in tissues or cells that have low or no endogenous PDE1 or PDE5 phosphodiesterase activity.

PDE9A1 is Most Homologous to RegA, a D. discoideum PDE—A search of GenBank using PDE9 sequence as a query indicates this PDE is slightly more homologous to a recently cloned D. discoideum PDE, RegA (3), than to any of the known PDEs. The search of GenBank using PDE9 sequence as a query indicates this PDE is slightly more homologous to a recently cloned D. discoideum PDE, RegA (3), than to any of the known PDEs.

**Inhibitor studies of PDE9 expressed in COS-7 cells**

| Inhibitor          | Selective for PDE type | $IC_{50}$ for PDE9 |
|--------------------|------------------------|--------------------|
| IBMX               | nonspecific (2–50 μM)  | >200               |
| Zaprinast          | PDE5/6 (0.7 sol. 15 μM)| 29                 |
| EHNA               | PDE2 (1 μM)            | >100               |
| Enoximene          | PDE2 (1 μM)            | >100               |
| Dipyridamole       | PDE5/6 (0.9/0.38 μM)   | >38                |
| SCH 51866          | PDE5/5 (0.1 μM)        | 1.55               |
| Sildenafil         | PDE5 (3.9 nM)          | 7                  |
| Rolipram          | PDE4 (2.0 μM)          | >200               |

**Fig. 5.** Hofstee plot of PDE9A1 kinetic assays. Assays were done as described under “Materials and Methods.” Substrate concentrations for this plot were 0.03, 0.04, 0.07, 0.13, 0.25, and 0.49 μM cGMP. $K_m$ is average of five separate assays on two different COS cell transfection batches. Plot is one representative experiment of the five assays. Maximal velocity was typically seen at 0.5 μM cGMP.

**Table I**

| Inhibitor          | Selective for PDE type | $IC_{50}$ for PDE9 |
|--------------------|------------------------|--------------------|
| IBMX               | nonspecific (2–50 μM)  | >200               |
| Zaprinast          | PDE5/6 (0.7 sol. 15 μM)| 29                 |
| EHNA               | PDE2 (1 μM)            | >100               |
| Enoximene          | PDE2 (1 μM)            | >100               |
| Dipyridamole       | PDE5/6 (0.9/0.38 μM)   | >38                |
| SCH 51866          | PDE5/5 (0.1 μM)        | 1.55               |
| Sildenafil         | PDE5 (3.9 nM)          | 7                  |
| Rolipram          | PDE4 (2.0 μM)          | >200               |
Cloning and Characterization of a Novel cGMP PDE Family

**Conclusion and Possible Physiologic Roles for PDE9A1—**

Although at this time it is unknown what physiologic roles PDE9 may regulate, the identification and initial description of this new family will aid in defining these roles in future studies. Because PDE9 is dedicated to the high affinity specific hydrolysis of cGMP, PDE9 must be involved in the regulation of signal transduction pathways that concern activation of guanylyl cyclase activity. For example, ANP receptors expressed in kidney regulate diuresis by ligand-activated receptor guanylyl cyclase activity (28–30). Because PDE9 is most highly expressed at the mRNA level in kidney, it is tempting to speculate that this novel PDE may participate in regulating this pathway by maintaining basal cGMP levels at low concentrations, by enlarging the ANP cGMP signal, and/or by lowering intracellular cGMP levels after ANP receptor activation, thus terminating ANP-mediated diuresis. Indeed, previous studies have shown that inhibition of cGMP hydrolysis by infusion of zaprinast (which at higher concentrations will inhibit PDE9; Table I) potentiates the effect of ANP on natriuresis without causing deleterious drops in blood pressure (31, 32). Because ANP receptors are known to be localized within the glomerulus and inner medullary collecting ducts (33), it will be interesting to determine the cellular localization of PDE9 enzyme in kidney.

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