The Calmodulin-dependent Phosphodiesterase Gene PDE1C Encodes Several Functionally Different Splice Variants in a Tissue-specific Manner*

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We report here the identification of cDNAs for three new mouse PDE1C splice variants and the characterization of their kinetics, regulation by Ca\(^{2+}\), sensitivities to inhibitors, and tissue/cellular expression patterns. Sequence analysis indicated that these three cDNAs (PDE1C1, PDE1C4, and PDE1C5), together with our previously reported PDE1C2 and PDE1C3, are alternative splice products of the PDE1C gene. The results from RNase protection analysis and in situ hybridization indicated that the expression of the different PDE1C splice variants is differentially regulated in a tissue/cell-specific manner. Particularly, high levels of PDE1C mRNAs were found in the olfactory epithelium, testis, and several regions of mouse brain such as cerebellar granule cells. All of these splice variants have similar kinetic properties, showing high affinities and approximately the same relative $V_{\text{max}}$ values for both cAMP and cGMP. However, they responded to Ca\(^{2+}\) stimulation differently. In addition, they showed different sensitivities to the calmodulin-dependent phosphodiesterase inhibitors, KS505a and SCH51886. Substrate competition experiments suggested the presence of only one catalytic site on these PDE1C isozymes for both cAMP and cGMP. In summary, these findings suggest that the PDE1C gene undergoes tissue-specific alternative splicing that generates structurally and functionally diverse gene products.

Hormones and neurotransmitters control the levels of the intracellular second messengers, cAMP and cGMP, by regulating the activity of cyclases and phosphodiesterases (PDEs). At least seven different families of PDE are currently recognized (1, 2). Most families contain several distinct genes, and many of these genes encode multiple alternative splice variants in a tissue-specific manner. The facts that different PDEs have unique sequences in their catalytic and/or regulatory domains and that they are often selectively expressed in a limited number of cell types allow cell-specific regulation of cyclic nucleotide level by the PDEs. They also provide a basis for selective therapeutic intervention.

The Ca\(^{2+}\)/calmodulin-dependent PDEs (CaM-PDEs) compose one of the best known of the multiple PDE families. All CaM-PDEs are activated by calmodulin in the presence of calcium. It is thought that CaM-PDEs act as mediators between the Ca\(^{2+}\) and cyclic nucleotide second messenger systems that allow cyclic nucleotide-dependent processes to be regulated by increases in intracellular Ca\(^{2+}\) concentration (2). A rather large family of CaM-PDE isozymes is expressed in mammals. At least six different members, including 59-, 61-, 63-, 68-, and 75-kDa as well as olfactory-enriched forms, have been described (3–8). These CaM-PDE isozymes are expressed in distinct cell types in various tissues and have different substrate specificities, specific activities, and activation characteristics by Ca\(^{2+}\) and CaM (9). To date, three different genes (PDE1A1, PDE1B1, and PDE1C1) have been identified in the CaM-PDE family. PDE1A1 and PDE1B1 have been extensively characterized (5, 10–12). Two splice variants of the PDE1A gene, PDE1A1 and PDE1A2, have been isolated from bovine heart and brain, respectively (10, 13). PDE1A1 and PDE1A2 encode the bovine heart 59-kDa and bovine brain 61-kDa CaM-PDE isozymes, respectively, and differ only in their N-termini (3). PDE1B1, which encodes the bovine brain 63-kDa CaM-PDE isozyme, has only one mRNA product isolated so far (5, 11, 12). PDE1C is a newly identified CaM-PDE gene, and one of its products (PDE1C2) is a dominant form of CaM-PDE present in olfactory sensory neurons (14). The genes encoding the brain 75-kDa and testis 68-kDa CaM-PDEs have not been identified.

We report here the isolation and characterization of three new splice variants of the PDE1C gene from a mouse brain library, which we call PDE1C1, PDE1C4, and PDE1C5. The protein sequences deduced from PDE1C1, PDE1C4, and PDE1C5 cDNAs are the same except in the C-terminal regions. However, they differ from the PDE1C2 protein sequence at both the N and C termini. More interestingly, PDE1C4 and PDE1C5 cDNAs have the same coding sequences but different 3’-untranslated regions (3’-UTRs), which is the first example of alternative splicing occurring solely in the 3’-UTR for mammalian PDEs. In order to understand the functional consequences of the different sequences among these variants, the kinetic properties, Ca\(^{2+}\) activation characteristics, inhibition by various CaM-PDE inhibitors, reciprocal inhibition between substrates, and the tissue-specific expression of multiple CaM-PDEs were systematically investigated. The data should help us understand the biological reasons for this great diversity and may provide a molecular basis for the design of selective inhibitors or activators of this PDE family.
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**EXPERIMENTAL PROCEDURES**

**Reverse Transcription-PCR and Cloning of PDE1C Products**—Using a reverse transcription-PCR approach, we obtained a PCR product that we named pIC1 (Fig. 1A) from a mouse brain first strand cDNA library purchased from Clontech. The PCR primers were 5′-TGGTACAGTGAGACTGCTGGGA-3′ (nucleotides 320–344) and 5′-ATGTCCTCATGTGATGAATT-3′ (nucleotides 863–887), corresponding to bovine PDE1A2 cDNA sequences that are conserved between bovine PDE1A and PDE1B (5, 10). The PCR reaction was carried out as follows: denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 1 min; for 30 cycles. The resulting amplified 585-nucleotide fragment was subcloned into the TA II vector (Invitrogen). Under blue white selection, the white colonies were picked and hybridized to 32P-labeled PDE1A2 and PDE1B1 cDNA probes. Several white colonies that did not hybridize to either PDE1A2 or PDE1B1 probes were further isolated and sequenced. This PCR fragment (pIC1) is homologous but different from PDE1A and PDE1B and was believed to represent a portion of a novel gene product.

**Cloning and Sequencing of PDE1C Splice Variants**—The PCR products, pIC1 and pIC1–2, were used as probes to screen a mouse brain 9.10 cDNA library (purchased from Clontech). Filters were washed at high stringency (0.1 × SSC buffer, 0.5% SDS at 65 °C). About 15 positive clones were detected from a total of 3 × 106 recombinant cDNA clones. Several of the CDNAs were excised with EcoRI digestion, subcloned into the Bluescript plasmid (Stratagene), and sequenced on both strands by constructing series of nested deletions using the Erase-A-Base system from Promega. Only one PDE1C4 clone was isolated from the brain library screening; and it lacked a 30-bp 5′-end of the open reading frame (ORF). The PCR4 clone shown in Fig. 1A is a composite with a whole ORF and was constructed by switching the 5′-end EcoRI-AccI fragment of the original PDE1C4 (lacking the first 30 bp of the ORF) with a corresponding PCR amplified fragment from PDE1C1.

**Transient Expression of Multiple PDE1C cDNAs and Assay for PDE Activity**—The PDE1.C1 (nucleotides 175–2252), PDE1C4 (nucleotides 8–2087), and PDE1C5 (nucleotides 521–2875) cDNAs with whole ORFs were subcloned into the multiple cloning region of the pcDNA3 vector (Invitrogen). The resulting plasmids, pcDNA3–1C1, pcDNA3–1C4, pcDNA3–1C5, and the previously constructed pcDNA3–1C2, pcDNA3–1A1, pcDNA3–1A2, and pcDNA3–1B1 (14) were used to transfect COS-7 cells with a calcium phosphate transfection kit (Invitrogen). Transfected cells were homogenized in a buffer containing 40 mM Tris-HCl (pH 7.5), 15 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 15 mM 2-mercaptoethanol using a Dounce homogenizer. Homogenates were supplemented with 20% glycerol and stored at −70 °C until use. Homogenates were assayed for PDE activity according to the method of Hansen and Beavo (15) in a buffer containing 100 mM MOPS (pH 7.2), 10 μM mossimazine (pH 7.5), 3 mM MgCl2, 15 mM magnesium acetate, 0.2 mg/ml of bovine serum albumin, and [3H]cGMP (100,000 cpm/tube) with either 400 μM EGTA or 200 μM C4 and 4 μg/ml of CaM or in a buffer (for the Ca2+ sensitivity studies) containing 50 mM MOPS (pH 7.2), 10 mM magnesium acetate (pH 7.2), 244 mM CaM, 1 μM cGMP, and [3H]GMP (100,000 cpm/tube). All PDE assay reactions were started by adding the enzyme into premixed and prewarmed other components. The CaM-PDE activities expressed in COS cells were calculated by subtracting the activities in mock-transfected cells from those in cDNA-transfected cells. For Ca2+ sensitivity studies, free calcium concentrations were varied using a CaCl2/EGTA buffer system and estimated using the Bound and Determined computer program (16). The total CaCl2 concentration was evaluated by the Calcium kit (reagents for the quantitative, colorimetric determination of calcium, from Sigma). The kinetic parameters were calculated from Eadie-Hofstee plots based on the best fit lines. Both Ca2+ activation curves and inhibitor curves were fitted with SigmaPlot 4.14 using a four-parameter logistic model. Inhibitor studies were performed at 0.1 μM cGMP, at which the observed IC50 approximates the apparent inhibitor constant (Ki). Stocks of inhibitors were prepared in MeSO, and the final solvent concentrations in the PDE assays were below 2%. Vinpocetine was purchased from Biorex; SCH51866 and KS505a were gifts from Schering-Plough Research Institute and Pharmaceutical Research Laboratories of Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan), respectively.

**Preparation of Polyclonal Antibody against PDE1C Recombinant Proteins**—A portion of the PDE1C2 C-terminal domain (amino acids 495–786) was fused in frame with the glutathione S-transferase moiety in the expression vector pGEX–4T–1 (Pharmacia Biotech Inc.). The cultured recombinant bacterial lysate was subjected to affinity chromatography using a glutathione Sepharose 4B column (Pharmacia Biotech Inc.), and the fusion protein was eluted with glutathione. The purified fusion protein was used in bacteria and eventually purified on glutathione affinity column according to a protocol from Pharmacia. Two rabbits were immunized with the purified glutathione S-transferase-PDE1C2 fusion protein. The resultant polyclonal antibody (α-PDE2-C) was tested on Western blot and recognized, as expected, all PDE1C isoforms.

**Western Blot Analysis of PDE1C Isomers Expressed in COS-7 Cells**—The COS-7 cell protein extracts containing various PDE1C splice variants were separated on an 8% SDS-polyacrylamide gel made from premixed gel solutions (National Diagnostics). The proteins were blotted onto nitrocellulose paper (Schleicher & Schuell) and reacted with the polyclonal antibody (α-C2-C). The specific PDE1C signals were detected using the Eastern chemiluminescent-horseradish peroxidase substrate system (Pierce).

**RNase Protection Assay**—RNase protection analysis was performed according to the protocol previously described (14). The riboprobe template pIC1 was derived from the 3′-UTR of PDE1C1 cDNA (see “In Situ Hybridization” and Fig. 1A). Western blot analysis was performed according to the protocol previously described (17). The riboprobe template pIC1–2 was synthesized based on the PDE1C1 cDNA (2417–2519) sequence and should be either fully or partially protected by PDE1C2, PDE1C4, or PDE1C5 transcripts. The predicted sizes of protected probe fragments are 192, 56, 116, and 179 bp for pIC1D1, PDE1C2, PDE1C4, and PDE1C5, respectively. Ten μg of total tissue RNA sample was applied to each assay. Each analysis included mouse β-mRNA as a control for the RNA quality and quantity among different tissue samples. The resultant autoradiogram was digitally recorded with a Hewlett-Packard image scanner and Deskscan II software. The intensity of the band of interest was measured with the NIH Image 1.58 program. The relative levels of each PDE1C splice variant were calculated by normalizing against the β-actin signal in the same tissue and corrected for the signal of protected bands. In Situ Hybridization—The procedures for in situ hybridization were performed as described previously (17). Mice were decapitated, and the brains were rapidly isolated, frozen in Tissue-Tek O.C.T. compound (Miles) on dry ice, and stored at −70 °C until use. Sections (14–16 μm) were cut in a cryostat, thaw-mounted onto a Vectabond reagent (Vector Laboratories)–treated microscope slide, fixed with 4% (w/v) paraformaldehyde, and stored at −70 °C.

The common region probe (pICN) recognizing all PDE1C splice variants was constructed from the PCR product initially used in library screening (Fig. 1A). The probes used to detect each different splice variant were derived from regions that are unique to each splice variant. The PDE1C1-specific probe (pIC1) is 192-bp long and is located in the 3′-UTR region of PDE1C1 cDNA (2061–2252) (Fig. 1A). The PDE1C2-specific probe (pIC2–1) is 361 bp long and in the 5′-region of PDE1C2 cDNA (1–261). The PDE1C5-specific probe (pIC5) is 352 bp long and in the 3′-UTR region of PDE1C5 cDNA (2531–2881). None of the regions is unique to PDE1C4 cDNA. A probe (pIC2–2) corresponding to the 3′-UTR region of PDE1C2 cDNA (2478–2886) is expected to hybridize to PDE1C2 and PDE1C4, and the probe (pIC4–1) should detect PDE1C2, PDE1C4, and PDE1C5. All of these riboprobe templates were constructed by PCR amplification of the corresponding regions of individual cDNAs and subcloning of the PCR products into TA II vectors (Invitrogen).

**Primary Culture of Mouse Cerebellar Granule Cells**—Primary cultures of cerebellar cells were prepared essentially as described by Bernard et al. (18) with minor modification. Cerebella were removed from 7-day-old mice, and the meninges were carefully removed. Each cerebellum was cut into small pieces and incubated for 10 min at 37 °C in Veronal (0.2 g/liter EDTA, from Life Technologies, Inc.). The cells were gently dissociated in serum-free medium by the confluence of use of four different sizes of needles with decreasing hole diameters (sizes: 18, 16, 20, and 22). Single cells were obtained by filtration through a cell strainer (Falcon) and plated (1.5–2.0 × 106 cells/ml, 5 ml/dish) in 60-mm-diameter Costar culture dishes, previously coated with 15 μg/ml poly-l-ornithine (molecular weight, 40,000; Sigma). The culture medium was composed of a 1:1 mixture of Dulbecco’s minimal essential medium and F-12 nutrient (Life Technologies, Inc.), supplemented with 30 mM glucose, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM
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RESULTS

Cloning and Sequencing PDE1C Splice Variants—Using a reverse transcription-PCR approach, we obtained a PCR product from mouse brain first strand cDNA that appeared to represent part of the PDE1C gene described previously (14). To isolate the cDNAs including the whole ORF in mouse brain, we initially screened a mouse brain cDNA library with the PCR product (p1CN) as a probe (Fig. 1A). Six clones were isolated from 1 million recombinant cDNA clones. Two distinct PDE1C splice variants with different 3'-ends were obtained from this screen. We designate them as PDE1C1 and PDE1C4, respectively.

In order to obtain additional clones, we PCR-amplified a fragment (p1C4–2) of the PDE1C4 3'-end (2417–2607) (see “Experimental Procedures”), which should be able to detect both PDE1C1 and PDE1C4. Using this probe, we screened an additional 2 million recombinant clones. Thirteen independent positive clones were isolated, eight of which represented PDE1C1 and three of which represented a new splice variant referred to as PDE1C5. Therefore, from the mouse brain library, we have obtained three distinct types of cDNAs (PDE1C1, PDE1C4, and PDE1C5), apparently resulting from alternative splicing of a single PDE1C gene depending on the position of the splice junction at the 3'-end.

Only one PDE1C4 clone was isolated from the brain library screening, and it lacked a 30-bp 5'-end of the ORF. To determine if the 5'-end of PDE1C4 clone was identical to that of PDE1C1, we performed reverse transcription-PCR using first strand cDNA synthesized from mouse brain mRNA. A DNA fragment was amplified in two rounds of PCR using two pairs of nested primers that were derived from the 5'-end of PDE1C1 ORF and 3'-end of PDE1C4 ORF (data not shown). The resulting DNA fragment has a PDE1C1 5'-end and PDE1C4 3'-end, indicating that PDE1C4 has the same 5'-end as PDE1C1.

Thus, we constructed a PDE1C4 clone with a whole ORF as shown in Fig. 1A (see “Experimental Procedures”).

In order to determine whether these distinct 3'-end sequences from the library are also present in a different source of mouse brain RNA, we were able to PCR-amplify each of the corresponding 3'-ends from first strand cDNA reverse-transcribed from mouse brain mRNA, indicating that the different 3'-end sequences are not likely to be library cloning artifacts (data not shown). In addition, consensus splice donor and acceptor sequences were not present in any splicing junctions within these 3'-ends (20), indicating that these various 3'-ends are not derived from intron sequences. This is also confirmed by the RNase protection results (Fig. 5).

Structural Analysis and Comparison of PDE1C Splice Variants—The translation initiation codons were determined by the RNase protection results (Fig. 5). Thus, we constructed a PDE1C4 clone with a whole ORF as shown in Fig. 1A (see “Experimental Procedures”).

As compared with the 3'-end DNA sequences of PDE1C2, PDE1C4 contains a 60-bp insertion, which introduces a stop codon and causes PDE1C4 to have a shorter C terminus than PDE1C2 (Fig. 1A). This 60-bp insertion does not contain consensus splice donor and acceptor sequences (20), suggesting that it is unlikely to be an intron. Analysis of PDE1C5 sequence

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FIG. 1. Multiple alternative splice variants of PDE1C gene. A, schematic representations of PDE1C1, PDE1C2, PDE1C4, and PDE1C5 cDNAs. Divergent sequences are indicated by patterns and stippling. The white boxes in PDE1C4 and PDE1C5 represent the 60-bp stretches mentioned in the text (see “Results”). The cDNA sequences for PDE1C1, PDE1C4, and PDE1C5 have been deposited in the GenBank data base (accession numbers are L76944, L76947, and L76946, respectively). The PDE1C2 cDNA was isolated and described previously (14). The PDE1C4 clone is a composite of the original PDE1C4 clone isolated from the mouse brain cDNA library and the missing region from PDE1C1. Regions of DNA fragment has a PDE1C1 5'-end and PDE1C4 3'-end, indicating that PDE1C4 has the same 5'-end as PDE1C1. Regions of the splice junction at the 3'-end DNA sequences of PDE1C2, PDE1C4, and PDE1C5 represent the 60-bp stretches shown in Fig. 1 (20), indicating that these various 3'-ends are not derived from intron sequences. This is also confirmed by the RNase protection results (Fig. 5).
revealed that PDE1C5 also contains this 60-bp stretch, and the sequence 5’ to the 60-bp stretch is identical to that of PDE1C4. However, the sequence 3’ to the 60-bp stretch is different.

The N terminus shared by PDE1C1 and PDE1C4/5 is different from that of PDE1C2 (Fig. 1C). The divergence point is located in the middle of the first CaM-binding domain identified in previous studies (3, 4) and corresponds to the alternative splice site that generates PDE1A1 and PDE1A2 (Fig. 1, C).

Expression of PDE1C Isozymes in COS-7 Cells—To determine whether the newly isolated PDE1C1, PDE1C4, and PDE1C5 cDNAs encoded biologically active CaM-PDEs, they were expressed in COS-7 cells by transient transfection as described previously for the expression of PDE1C2 (14). Little CaM-PDE activity was detected in the mock cells that were transfected with pcDNA3 vector DNA, indicating that COS-7 cells have very low endogenous CaM-PDE activity. However, Ca\(^{2+}\) and CaM cause a 6–7-fold stimulation of cAMP or cGMP hydrolysis in homogenates from PDE1C1 cDNA-transfected cells (Fig. 2A), indicating that these PDE1C splice variants encode functionally active CaM-PDEs. The integrity and the molecular weight of expressed PDE1C proteins were examined by Western blot analysis with the polyclonal antibody α-1C2-C (Fig. 2B). A single band with a molecular weight close to that predicted was detected for each PDE1C cDNA-transfected cell extract.

Kinetic Properties of Multiple PDE1C Splice Variants—To determine whether the different splice variant coding sequences have any influence on enzymatic properties, the effect of substrate concentration on the initial velocity of cyclic nucleotide hydrolysis was examined. The \(K_m\) and \(V_{max}\) ratio for cAMP and cGMP are summarized in Table I. The \(K_m\) values of PDE1C1 for cAMP and cGMP are 3.5 \(\mu\)M and 2.2 \(\mu\)M, respectively, slightly higher than those of PDE1C2. The \(K_m\) value of PDE1C4 or PDE1C5 for either cAMP or cGMP is close to 1 \(\mu\)M, very similar to that of PDE1C2. The \(V_{max}\) ratio is approximately 1 for both PDE1C1 and PDE1C4/5, which are the same as that for PDE1C2. The slightly higher \(K_m\) values of the PDE1C1 isozyme may be determined by its unique C terminus.

\(Ca^{2+}\) Activation of the Multiple CaM-PDEs—The \(Ca^{2+}\) sensitivity of different CaM-PDEs expressed in COS-7 cells has been examined and is summarized in Fig. 3. PDE1A1 and PDE1A2 are two splice variants of the PDE1A gene with different N termini. A putative Ca\(^{2+}\)/CaM binding domain was identified near the divergence point of their N termini (Fig. 1C). It has been found that the biochemically purified bovine 59-kDa (PDE1A1) and 61-kDa (PDE1A2) CaM-PDEs show substantial differences in response to Ca\(^{2+}\) or CaM stimulation (21, 22). Therefore, we used COS-7 cell-expressed recombinant bovine PDE1A1 and PDE1A2 as positive controls for this assay and found that the half-maximal stimulation of CaM-PDE activity (EC\(_{50}\)) for PDE1A1 was at 0.27 \(\mu\)M free Ca\(^{2+}\), whereas that for PDE1A2 was at 1.99 \(\mu\)M, consistent with previously reported values for purified enzymes. Among PDE1C splice variants, PDE1C1 and PDE1C4/5 have the same N termini and show similar \(Ca^{2+}\) sensitivities; their EC\(_{50}\) values for Ca\(^{2+}\) are 3.01 and 2.43 \(\mu\)M, respectively. However, PDE1C2 shows a different \(Ca^{2+}\) sensitivity; its EC\(_{50}\) is 0.83 \(\mu\)M. Since the difference in N termini of PDE1A1 and PDE1A2 accounts for their disparity of \(Ca^{2+}\) sensitivity, we speculate that the unique N terminus of the PDE1C2 might be the main determinant of its higher sensitivity to \(Ca^{2+}\) stimulation than those of other PDE1C variants. However, currently we cannot rule out the possibility that the C-terminal domain of PDE1C2 might also contribute to its unique \(Ca^{2+}\) sensitivity. Overall, our results indicate that the different PDE1C splice variants have different sensitivities to \(Ca^{2+}\).

**Table I**

| Isozymes      | \(K_m\) \(\mu\)M | \(V_{max}\) \(\mu\)M | \(V_{max}\) ratio (cAMP/cGMP) |
|---------------|------------------|----------------------|-------------------------------|
| Bovine PDE1A2 | 112.7 ± 7.9      | 5.1 ± 0.6             | 2.9 ± 0.1                     |
| Bovine PDE1B1 | 24.3 ± 2.9       | 2.7 ± 0.2             | 0.9 ± 0.1                     |
| Mouse PDE1C1  | 3.5 ± 0.3        | 2.2 ± 0.1             | 1.3 ± 0.1                     |
| Rat PDE1C2    | 1.2 ± 0.1        | 1.1 ± 0.2             | 1.2 ± 0.1                     |
| Mouse PDE1C4/5| 1.1 ± 0.0        | 1.0 ± 0.1             | 1.0 ± 0.0                     |

**Fig. 3. Comparison of \(Ca^{2+}\) sensitivity among multiple CaM-PDEs**—Different CaM-PDEs were transiently expressed in COS-7 cells and assayed for PDE activity in the presence of 4 \(\mu\)g/ml CaM. Free Ca\(^{2+}\) concentrations were varied using EGTA/CaCl\(_2\) buffers as described under “Experimental Procedures.” PDE activities are the mean of triplicate determinations. The EC\(_{50}\) of \(Ca^{2+}\) stimulation for each CaM-PDE was the free Ca\(^{2+}\) concentration at which the CaM-PDE activity reached the half-maximum. Results are expressed as mean ± S.D. of at least three independent experiments performed in triplicate.
inhibited PDE1A and PDE1B isoforms with similar potencies, which are about 3-fold higher than that for PDE1C isoforms. Vinpocetine appeared not to be able to distinguish between splice variants.

KS505a has been shown to inhibit a bovine brain CaM-PDE preparation with much higher potency than that for a heart enzyme, probably by binding near the CaM-binding site of the enzyme (24). In the presence of 244 nM CaM, KS505a showed about 8-fold higher potency for PDE1A2 (the bovine brain form) than for PDE1A1 (the bovine heart form). In addition, among the PDE1C isoforms, KS505a also exhibits higher (about 3-fold) potency for PDE1C1 or PDE1C4/5 than that for PDE1C2. CaM did not seem to compete with the KS505a for any of the CaM-PDE tested because 10-fold lower CaM did not cause any shift of the inhibition curves (data not shown). The fact that KS505a has different potencies for the splice variants with distinct N termini suggests that KS505a interacts with the N-terminal regions of the CaM-PDEs.

SCH51866 is a compound that has been reported to selectively inhibit the CaM-PDEs and cGMP-binding PDEs (25). In our studies, SCH51866 showed similar potencies for PDE1A1 and PDE1A2, which were much higher than that for PDE1B1. Interestingly, in the PDE1C subfamily, SCH51866 has almost the same potencies for PDE1C2 and PDE1C4/5 but about 3-fold lower potency for PDE1C1. This result indicates that the differences in SCH51866 inhibition are probably determined by the catalytic and C-terminal regions of the enzymes. In summary, the results demonstrate that these three CaM-PDE inhibitors have different selectivity profiles and that they are able to distinguish between some subfamily members and even between splice variants.

**Reciprocal Inhibition between Substrates**—To establish whether the PDE1C isoforms form distinct catalytic sites for the two substrates, cAMP and cGMP, the effect of different concentrations of the nonradioactive cyclic nucleotide on the hydrolysis of different concentrations of the labeled cyclic nucleotide as a substrate (S). A, substrate is cAMP; and inhibitor is cGMP. B, substrate is cGMP, and inhibitor is cAMP. Parallel lines indicate competitive inhibition. Each point is the mean of triplicates.

**Table II**

| Isozymes          | IC50 values (µM) | Km values (µM) | Vmax values (10^5 x 10^3) |
|-------------------|------------------|----------------|--------------------------|
|                   | Vinpocetine      | KS505a         | SCH51866                 |
| Bovine PDE1A1     | 9.7 ± 0.2        | 55.9 ± 2.8     | 13.0 ± 1.4               |
| Bovine PDE1A2     | 10.9 ± 1.0       | 6.9 ± 0.8      | 18.0 ± 2.3               |
| Bovine PDE1B1     | 9.8 ± 0.9        | 7.9 ± 0.6      | 68.0 ± 3.9               |
| Mouse PDE1C1      | 39.5 ± 3.5       | 2.5 ± 0.5      | 101.2 ± 3.2              |
| Rat PDE1C2        | 32.4 ± 3.9       | 7.5 ± 0.5      | 33.5 ± 2.3               |
| Mouse PDE1C4/5    | 42.4 ± 2.6       | 2.3 ± 0.3      | 36.0 ± 4.6               |

**Fig. 4.** Hanes-Woolf plots of reciprocal inhibition between substrates. This figure shows the effects of different concentrations of the nonradioactive cyclic nucleotide as an inhibitor (I) on the hydrolysis of different concentrations of the labeled cyclic nucleotide as a substrate (S). A, substrate is cAMP; and inhibitor is cGMP. B, substrate is cGMP, and inhibitor is cAMP. Parallel lines indicate competitive inhibition. Each point is the mean of triplicates.
splice variants. The intensities of different variant-specific \(^{32}\)P-labeled riboprobes (see "Experimental Procedures"). The levels of alternatively spliced PDE1C variants exhibit distinctive tissue distribution patterns and that the expression of PDE1C in some tissues can be species-dependent.

Regional and Cellular Expression of PDE1C Splice Variants in Mouse Brain by in Situ Hybridization—To examine in more detail the cell type-specific expression of PDE1C mRNAs in the mouse brain, we used in situ hybridization with the \(^{35}\)S-labeled riboprobes generated from regions either common to all types of PDE1C cDNAs or specific to each splice variant (see "Experimental Procedures").

Fig. 6 shows dark field digital images of three representative sections hybridized with the common region probe (p1CN), which detects all PDE1C mRNAs. The PDE1C gene has a unique expression pattern that is different from those of the PDE1A and PDE1B genes (17). PDE1C mRNAs were highly concentrated in the neurons of the granule layer of the cerebellum, some Purkinje cells, the central amygdaloid nucleus, and the interpolar spinal trigem nucleus. Moderate levels of PDE1C mRNAs are found in the glomerular and external plexiform layer of the olfactory bulb as well as in parts of the caudate-putamen and olfactory tubercle.

In order to determine whether the different PDE1C splice variants were expressed in different types of cells, in situ hybridization experiments using specific probes were performed. Fig. 7 illustrates high magnification dark field digital images of five representative regions hybridized with the common region probe p1CN (panels a–c), the PDE1C1-specific probe p1C1 (panels d–f), or the PDE1C5-specific probe p1C5 (panels g–i). In general, PDE1C5 mRNA showed a more limited localization than PDE1C1 mRNA. In the olfactory bulb, both PDE1C1 and PDE1C5 mRNAs are localized in periglomerular cells of the glomerular layer and in the tufted cells of the external plexiform layer, although the PDE1C5 \(\alpha\)C probe gave a less intense hybridization signal than the PDE1C1 probe. Similarly, in the central amygdaloid nucleus, both PDE1C1 and PDE1C5 mRNAs are detected. In the caudate-putamen and olfactory tubercle of the mouse forebrain, as well as the interpolar spinal trigem nucleus of the brain stem, a significant amount of PDE1C1 mRNA is expressed; however, the PDE1C5 mRNA level is below the level of detection. In the cerebellum, PDE1C1 mRNA is expressed in both granular cells and a subset of Purkinje cells. In contrast, PDE1C5 is expressed in the granule cells but not Purkinje cells. A PDE1C2-specific probe (p1C2–1, see Fig. 1A) has also been examined; no significant hybridization signal was detected in any regions of the mouse brain (data not shown), which is consistent with the result from the RNase protection assay (Fig. 5). A PDE1C4-specific probe could not be created, because there is no unique sequence existing in PDE1C4 cDNA. Although the PDE1C4 clone was isolated from a mouse brain library, the average level of PDE1C4 mRNA must be very low in mouse brain. This is supported not only by the observation of a very weak band for PDE1C4 mRNA in mouse brain tissue from the RNase protection assay (Fig. 5), but also by the library screening in which only one clone has been isolated from 3 \(\times\) 10\(^5\) recombinants. The number of cDNA clones for each species of mRNA in the library is likely to reflect the relative abundance of each species of mRNA. In addition, when we used the probe, p1C4 or p1C2–2, which would detect PDE1C2 (14), PDE1C4, and PDE1C5, the hybridization pattern or signal intensity was equivalent to that with the PDE1C5-specific probe alone (data not shown), indicating that the PDE1C4 mRNA level is very low in mouse brain.

CaM-PDE Activity in Primary Cultured Cerebellar Granule Cells—In situ hybridization results suggest that both PDE1C1 and PDE1C5 are expressed in cerebellar granule cells (Figs. 6 and 7). Therefore, we carried out PDE assays on primary cultured cerebellar granule cells in order to determine whether cerebellar granule cells indeed retain high levels of high affinity CaM-PDE activity derived from the PDE1C gene. Cyclic nucleotide hydrolysis was measured at substrate concentrations ranging from 0.1 to 300 \(\mu\)M. The kinetic data fit best with a double rectangular hyperbolic model, suggesting that there are two CaM-PDE activities with different kinetic constants in the granule cell extracts. The major CaM-PDE activity had kinetic constants for cAMP and cGMP similar to that of ex-
pressed PDE1C1 and PDE1C5. The results indicate that cultured granule cells contain a large amount of CaM-PDE activity with a high affinity for both cAMP and cGMP, which is consistent with our previous observation that PDE1C, and not PDE1A or PDE1B, is the major and perhaps only type of CaM-PDE highly expressed in the cerebellar granule cells (Figs. 6 and 7, Ref. 17). More importantly, these results suggest that such neuronal cultures can be used for studying the potential role of PDE1C enzymes in Ca$^{2+}$-mediated regulation of intracellular cyclic nucleotide levels in cerebellar granule cells.

**DISCUSSION**

We report here the isolation and characterization of three new mouse PDE1C cDNA clones named PDE1C1, PDE1C4, and PDE1C5. Taken together with other recent data, it now appears that alternative splicing of the PDE1C gene generates at least five distinct mRNAs: PDE1C1, PDE1C2 (14), PDE1C3 (26), PDE1C4, and PDE1C5. Analysis of the genomic sequence of the PDE1C gene will be required to reveal the detailed mechanisms for generating these multiple species of PDE1C mRNA.

PDE1C1 and PDE1C4/5 share the same N terminus, which is different from that of PDE1C2. The divergence point is located within the first calmodulin binding domain identified in PDE1A1 and PDE1A2 (3, 4) and at a very similar relative position to the alternative splicing site that yields PDE1A1 and PDE1A2 (Fig. 1C). Similar to the differences in Ca$^{2+}$ sensitivity between PDE1A1 and PDE1A2, PDE1C1 and PDE1C4/5 have much lower Ca$^{2+}$ sensitivity than PDE1C2 (Fig. 3). This result further supports the idea that the N terminus of the CaM-PDE is the region where Ca$^{2+}$/calmodulin binds. In addition, this PDE1C example and the PDE1A example strongly suggest that the functional differences (e.g. Ca$^{2+}$ activation) among CaM-PDE isozymes can be attributed to their structural diversity and that alternative splicing provides an additional mechanism for increases in functional and structural diversity.

PDE1C4 and PDE1C5 cDNAs differ only in their 3′-UTRs and thus encode the same protein. PDE1C1 and PDE1C4/5 have the same N termini and different C termini. The kinetic properties and Ca$^{2+}$ sensitivity of these enzymes with different C termini are very similar, suggesting that the C-terminal sequences are not important for the enzyme’s catalytic activity nor the interaction with Ca$^{2+}$/calmodulin. But it is not yet known whether these different C-terminal sequences have any consequences for stability, intracellular localization, regulatory modification, or cell-specific expression. The PDE1C4 and PDE1C5 cDNAs appear to be generated by alternative splicing of 3′-UTRs. Much experimental evidence indicates that sequences in 3′-UTRs critically influence post-transcriptional regulation of mRNAs during growth, differentiation, and response to environmental stimuli (27–30). Hence, the functional role for the alternatively spliced 3′-UTRs of PDE1C4 and PDE1C5 may be in post-transcriptional, translational, and/or tissue-specific regulation of the gene expression. Clearly, more
work is required to define the functional role for the unique sequences found in the different splice variants.

Although a number of compounds are capable of inhibiting the CaM-PDEs, most of them lack selectivity between CaM-PDE isozymes. The three compounds tested in this study showed some selectivity between CaM-PDE isozymes and even between splice variants. The fact that these three compounds have different selectivity profiles may reflect different mechanisms of inhibition. Availability of such selective inhibitors should not only provide useful tools for understanding biological function of each isozyme but also offer an initial starting point for development of agents having higher selectivity.

CaM-PDEs with high affinities for both cAMP and cGMP have been described in various tissues, such as olfactory epithelium (8), testis (6), heart (31), and pancreas (32). PDE1C2 is likely to represent the high affinity CaM-PDE activity in olfactory epithelium (8, 14). The data presented in this paper help to define which gene products are responsible for some of the high affinity CaM-PDE activities described in previous studies. A mouse testis enriched 68–70-kDa CaM-PDE that exhibits micromolar affinity for both cAMP and cGMP has been purified 900-fold and characterized previously (6). The present data show that PDE1C1 and PDE1C4/5 isozymes are highly expressed in the mouse testis and have high affinities to both cAMP and cGMP as well as molecular masses close to 70 kDa. These features suggest that it is very likely that some combination of these PDE1C isozymes represents the high affinity CaM-PDE hydrolytic activity seen in the testis CaM-PDE preparation. However, two differences in biochemical parameters did not seem consistent with this conclusion. First, the purified testis enzyme is suggested to have two distinct catalytic sites, because each of the two substrates, cAMP and cGMP, acts as a noncompetitive inhibitor of the other. However, the PDE1C isozymes expressed in this study appear to have only one catalytic site for both substrates (Fig. 4). Second, the purified testis enzyme showed both high and low affinity kinetic components for both cAMP and cGMP. However, the expressed PDE1C isozymes have only the high affinity component. It seems likely that the apparent discrepancy between the expressed PDE1C activity in this study and the previously reported testis enzyme could be best explained by the assumption that the testis enzyme preparation used in the early studies may have contained more than one CaM-PDE, at least one of which has a lower affinity for cAMP and cGMP.

High levels of specific PDE isozyme expression are often associated with specific functions of the region, such as the cGMP-specific PDE for visual transduction cascade in the retina (33) and the cGMP-stimulated PDE for the aldosterone production in the adrenal cortex (34). Recently, in cytosolic fractions of purified synaptosomes, the breakdown of cGMP was found to be highly stimulated by Ca²⁺ (35). Therefore, it was proposed that a CaM-PDE is likely to be involved in preventing elevations of intracellular cGMP levels in activated, nitric oxide-producing granule cells (35). It seems likely that this CaM-PDE activity may be contributed by PDE1C1 and PDE1C5. The fact that PDE1C1 and PDE1C5 represent the major CaM-PDE activities in granule cells of cerebellum suggests an important role for these PDEs in this down-regulation of cGMP of granule cells seen in response to excitatory amino acid stimulation. Primary cultured cerebellar granule cells exhibiting a high level of PDE1C activity should be a good system for further study of the physiological function of PDE1C in the granule cells.

At least three CaM-PDE genes and alternative RNA processing pathways generate an unexpectedly large diversity of CaM-PDEs. More than one CaM-PDE or different splice variants are often expressed in the same tissue or even in the same cell types. Therefore, the kinetic constants or the results of inhibitor analysis determined by using biochemically purified enzymes from many tissues are often complicated by the contamination of one or more closely related enzymes. Our efforts in molecular cloning and expression of individual CaM-PDE have allowed us to define the kinetic characteristics of single isoforms. In addition, it is likely that the identification and characterization of the new PDE1C splice variants reported here and previously will aid in understanding the functional consequences resulting from unique CaM-PDE expression as well as provide molecular basis for the development of isozyme-selective CaM-PDE inhibitors.

REFERENCES

1. Beavo, J. A., Conti, M., and Heaslip, R. J. (1994) Mol. Pharmacol. 46, 399–405
2. Beavo, J. A. (1995) Physiol. Rev. 75, 725–748
3. Novack, J. P., Charbonneau, R., Bentley, J. K., Walsh, K. A., and Beavo, J. A. (1991) Biochemistry 30, 7940–7947
4. Charbonneau, H., Kumar, S., Novack, J. P., Blumenthal, D. K., Griffin, P. R., Shabanowitz, J., Hunt, D. P., Beavo, J. A., and Walsh, K. A. (1991) Biochemistry 30, 7931–7940
5. Bentley, J. K., Kadlecak, A., Sherbert, C. H., Seger, D., Sonnenburg, W. K., Charbonneau, H., Novack, J. P., and Beavo, J. A. (1992) J. Biol. Chem. 267, 18676–18682
6. Rossi, P., Giorgi, M., Geremia, R., and Kincade, R. L. (1988) J. Biol. Chem. 263, 15521–15527
7. Shenolikar, S., Thompson, W. J., and Strada, S. J. (1985) Biochemistry 24, 672–678
8. Borisy, F. F., Ronnett, G. V., Cunningham, A. M., Juilfs, D., Beavo, J. A., and Snyder, S. H. (1992) J. Neurosci. 12, 915–923
9. Beavo, J. and Houlay, M. D. (1990) Cyclic Nucleotide Phosphodiesterases: Structure, Function, Regulation, and Drug Action, Vol. 2, pp. 19–46, John Wiley & Sons, Chichester, United Kingdom
10. Sonnenburg, W. K., Seger, D., and Beavo, J. A. (1993) J. Biol. Chem. 268, 645–652
11. Repaske, D. R., Swinnen, J. V., Jin, S.-L. C., Van Wyck, J. J., and Conti, M. (1992) J. Biol. Chem. 267, 18683–18688
12. Polli, J. W., and Kincaid, R. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11079–11083
13. Sonnenburg, W. K., Seger, D., Kwak, K. S., Huang, J., Charbonneau, H., and Beavo, J. A. (1995) J. Biol. Chem. 270, 30989–31000
14. Yan, C., Zhao, A. Z., Bentley, J. K., Loughney, K., Ferguson, K., and Beavo, J. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9677–9681
15. Hansen, R. S., and Beavo, J. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 79, 2788–2792
16. Brooks, S. P., and Storey, K. B. (1992) Anal. Biochem. 201, 119–126
17. Yan, C., Bentley, J. K., Sonnenburg, W. K., and Beavo, J. A. (1994) J. Neurosci. 14, 973–984
18. Van, Y. B. J., Sebben, M., Dumuis, A., Gaboron, J., Boekart, J., and Pin, J. P. (1989) J. Neurochem. 52, 1229–1239
19. Didier, M. R., Pou, P., Pichaczek, T., Managart, P., Devillers, G., Beokart, J., and Pin, J. P. (1992) Brain Res. Mol. Brain Res. 12, 249–258
20. Shapiro, M. B., and Senapathy, P. (1987) Nucleic Acids Res. 15, 7155–7174
21. Hansen, R. S., and Beavo, J. A. (1986) J. Biol. Chem. 261, 14636–14645
22. Sharma, R. K. (1991) Biochemistry 30, 5963–5968
23. Haywara, M., Endo, T., and Hidaka, H. (1984) Biochem. Pharmacol. 33, 455–457
24. Nakanishi, S., Osawa, K., Saito, Y., Kawamoto, I., Kuroda, K., and Hase, H. (1992) J. Antibiot. (Tokyo) 45, 341–347
25. Watkins, R. W., Davis, H. R., Jr., Fawzi, A., Ahn, H. S., Cook, J., Cleven, R., Hood, L., McGregor, D., McElroy, P., Pala, K., Tedesco, R., and Tulsiani, D. (1995) FASEB J. 9, 342 (abstr.)
26. Loughney, K., Martins, T., Sonnenburg, B., Beavo, J., and Ferguson, K. (1994) Biochem. J. 9, 469 (abstr.)
27. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667
28. Brawerman, G. (1987) Cell 48, 5–6
29. Jackson, R. J., and Standart, N. (1990) Cell 62, 15–24
30. Gillis, P., and Malter, J. S. (1981) J. Biol. Chem. 256, 3172–3177
31. Reves, M. L., Leigh, B. K., and England, P. J. (1987) Biochem. J. 241, 553–541
32. Vandermeers, A., Vandermeers, P. M. C., Rathie, J., and Christophe, J. (1963) Biochem. J. 3, 341–347
33. Fung, B. K., Hurley, J. B., and Stryer, L. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 152–156
34. Mayer, L. T., Zelus, B. D., and Beavo, J. A. (1991) J. Biol. Chem. 266, 136–142
35. Mayer, B., Klett, P., Bohme, E., and Schmidt, K. (1992) J. Neurochem. 92, 2024–2029