Isolation and Characterization of cDNAs Corresponding to Two Human Calcium, Calmodulin-regulated, 3',5'-Cyclic Nucleotide Phosphodiesterases*

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Cyclic nucleotides are involved in a large number of mammalian signal transduction pathways. The intracellular concentrations of cAMP and cGMP reflect their rate of synthesis, by adenylyl and guanylyl cyclases, and their rate of degradation to 5'-monophosphate nucleosides, by cyclic nucleotide phosphodiesterases (PDEs). A number of biochemically distinct PDEs have been identified. They fall into seven families distinguished by their allosteric regulation, substrate kinetics, amino acid sequence homology, and interaction with specific inhibitors (1, 2). All known mammalian PDEs share a conserved region of approximately 250 amino acids that contains the phosphodiesterase catalytic site (3). In certain PDEs the regions amino-terminal to the catalytic domain are known to be involved in the allosteric regulation caused by the binding of calmodulin or cGMP (3).

Type I PDEs (PDE1, CaM PDEs) are activated by the binding of calmodulin in the presence of Ca²⁺ (CaM). This binding increases the hydrolysis of both cAMP and cGMP (4). The regulation of cyclic nucleotide hydrolysis by changes in calcium concentration may allow the type I enzymes to integrate the Ca²⁺ and cyclic nucleotide second messenger pathways within a cell.

Biochemical characterizations have distinguished at least five type I PDEs differing from each other in apparent molecular weight, Km values for cyclic nucleotide substrates, affinities for calmodulin, and regulation by phosphorylation (5–16).

The cDNAs for several of these type I PDEs have been isolated and characterized (17–20). The existence of type I PDEs with distinct sequences and properties suggests a diversity of cellular functions for these enzymes. However, this diversity complicates the analysis of type I PDEs in tissues where more than one biochemical form is present. Isolating each of the type I PDE genes helps aid in understanding the role played by each member of this complex family of enzymes.

We report here the isolation and characterization of cDNAs corresponding to two human type I PDE genes. One, Hcam1, corresponds to the bovine 61-kDa CaM PDE. The other, Hcam3, is a novel type I PDE, whose mouse and rat counterparts have also been recently isolated (52). We examine the tissue distribution of the mRNAs for Hcam1 and Hcam3 and the biochemical properties of amino-truncated proteins corresponding to each gene. Hcam3 shows alternative splicing that corresponds to each gene. Hcam3 shows alternative splicing that differs most notably from the biochemical properties of amino-truncated proteins corresponding to each gene. Hcam3 shows alternative splicing that.

MATERIALS AND METHODS

Library Screening—A bovine 61-kDa CaM PDE cDNA fragment was used as a probe to screen human cDNA libraries. Degenerate oligonucleotides (corresponding to the bovine 61-kDa CaM PDE 3' PDE, phosphodiesterase; CaM, calmodulin; PCR, polymerase chain reaction; RT, reverse transcription; PIPES, 1,4-piperazinediethanesulfonic acid; IBMX, isobutylmethylxanthine; bp, base pair(s); kb, kilobase pair(s).
amino acid sequences KGMGMMK and NMGGTNTD and bovine heart cDNA were used in a PCR reaction to generate an 1108-bp bovine cDNA fragment as previously described (17). Hybridization probes were prepared by isolating DNA fragments from agarose gels and labeling them with [32P]dCTP and [32P]dTTP (800 Ci/mmol, DuPont) by using a Boehringer Mannheim random priming kit. Library screening and hybridization conditions were as described elsewhere (21). 3′ and 5′ end fragments were used in the primary reaction, and primers 1 and 2 were used to generate the cDNA inserts from that page which were subsequently used as probes to isolate additional ϕ phage. An Hϕ probe (1.2-kb HindIII/ECORV fragment) was used to isolate additional λ phage and Aϕ2d from an aorta cDNA library (Clontech). The cDNA inserts from these phage were subsequently used as probes to isolate additional λ phage. An Hϕ probe (1.2-kb HindIII/ECORV fragment) was used to isolate additional λ phage and Aϕ2d from an aorta cDNA library (Clontech). An Hϕ3a probe (2.4-kb HindIII/ECORI fragment) was used to isolate additional λ phage, He1lα and He1lαa, from the heart library (Stratagen).

Subcloning and DNA Sequencing—cDNA inserts from Hϕa, Hϕ2, Hϕ3a, and Aϕ2d were subcloned into Bluescript vectors (Stratagen). Bluescript plasmids containing the cDNA inserts from He1lα and He1lαa were excised in vivo from the λ ZAP vector (Stratagen) following the manufacturer’s instructions. Plasmid DNA was extracted using kits from Promega Biotech Inc. and QIAGEN Inc. Restriction and modification enzymes were purchased from Boehringer Mannheim. Oligonucleotides were synthesized using an ABI 394 DNA synthesizer. The cDNA inserts from Hϕa, Hϕ3a, He1lα, and Aϕ2d were completely sequenced on both strands using a Sequenase kit (U. S. Biochemical Corp). Those from He1lαa and Hϕ2a were partially sequenced. Additional molecular biological techniques are as previously described (22).

RNA Isolation—Human tissue samples were obtained from the National Disease Research Interchange and the Cooperative Human Tissue Network. Tissue was frozen within 8 h of death or surgery except for three of the four heart samples which were frozen within 30 h of death. The tissues were pulverized under liquid nitrogen, and RNA was extracted by the method of Chomczynski and Sacchi (23).

RT-PCR—First strand cDNA was reverse-transcribed from 2 μg of human heart poly(A)+ mRNA using a Boehringer Mannheim cDNA synthesis kit. 0.5 μl of the 20-μl final reaction volume was used per subsequent PCR reaction, which also contained 10 μg/ml of each primer, 10 μM Tris, pH 8.3, 1.5 μM MgCl2, 50 μM KCl, 0.2 μM each dNTP (Boehringer Mannheim), 0.6 unit Taq polymerase (Boehringer Mannheim) in 25 μl. Reaction conditions were 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, and 60°C for 2 min and 72°C for 4 min. The polymerase was added after the reaction temperature reached 94°C. Primary PCR reactions were diluted 1:100, and 1 μl was added to a 25-μl secondary PCR reaction. Hcam1 primers are shown in Fig. 2, A and C; primers 1 and 4 were used in the first reaction and primers 2 and 3 in the secondary reaction. Hcam3 primers are shown in Fig. 2, B and C; primers 1 and 4 were used in the first reaction and primers 2 and 3 in the secondary reaction. The PCR-derived DNA fragments were gel purified and partially sequenced.

RNA Protection Assays—An antisense Hcam1 RNA probe (made from a Hcam1-containing cDNA 252–1993; Fig. 1B) was prepared by in vitro transcription using 20 ng of a linearized template. The transcription reaction was performed at 37°C for 30 min and contained 40 μM Tris-HCl, pH 8, 50 μM NaCl, 8 μM MgCl2, 2 μM spermidine, 0.25 mM each ATP, CTP, and GTP, 0.1 unit of RNase block I (Stratagen), 5 μM dithiothreitol, 50 μM UTP, 5 μM a-[32P]UTP (800 Ci/mmol, DuPont), and 10 units of T7 RNA polymerase (Stratagen) in a reaction volume of 5 μl. Escherichia coli RNA (30 μg) and 5 μl of 40 μM Tris-HCl, pH 8.6, 6 μM MgCl2, and 10 mM NaCl were added prior to treatment with 10 units of RNase-free DNase (Boehringer Mannheim) for 15 min at 37°C. The reaction was extracted sequentially with phenol/chloroform and chloroform and then precipitated (50 μl of 7.5% ammonium acetate, 300 μl of ethanol).

RNA protection conditions were based on published methods (24). The probe was resuspended in 80% formamide, 400 mM NaCl, 40 mM Pipes pH 7, and 1 μl EDTA. Human total RNA (4–10 μg in 1.7–8.9 μl) was added to 50-μl aliquots of the probe (5 × 10^6 dpm), denatured at 95°C for 5 min and then incubated with 1 unit/ml RNase T1 (Sigma) for 30 min at 30°C. The reaction mixture was diluted 10-fold with 50 mM NaCl, 20 mM Pipes pH 6.8, 5 × SSC, 1 μl EDTA, 2.5 μl Denhardt’s solution, 200 μg/ml denatured salmon testes DNA, 0.5 mg/ml RNA, and 20 units/ml heparin. The RNase protection analysis. The Hcam3a-specific probe extended from nucleotides 2068–2686 (Fig. 2C) and the Hcam3b-specific probe extended from nucleotides 2062–2652 (Fig. 2B). Fragments corresponding to these regions were generated by PCR and subcloned prior to their use in preparing an antisense riboprobe (as described above).

Expression Constructs—The cDNAs were cloned into the yeast expression vector pBNY6n and expressed in Saccharomyces cerevisiae. This vector, which provided EcorI and XhoI cloning sites, was constructed from pADNS (25) and Yeplac181 (26) as follows. The polylinker of pADNS was replaced with an EcoRl/NotI/XhoI linker (5′-ACCTGGATATCTTGGGCGGCGGGCGGCGGCGGGCAATTC-3′). This linker was ligated into pADNS that had been cut to completion with HindIII and partially digested with EcoRl. The resultant plasmid was cloned with BamHI and the fragment that contained the ADH promoter and terminator flanking the new polylinker was inserted into the BamHI site of a Yeplac181 derivative. In this derivative the EcorI site of Yeplac181 had been removed by digestion with HpaI and Smal followed by ligation.

The plasmid Hcam3a (met150) was constructed by ligating the cDNA insert of Hiel1α (nucleotides 610–2067 of Fig. 2B) followed by 2068–2686 of Fig. 2C) into the EcorI site of pBNY6n. New junctions in the construct were verified by DNA sequencing. The first ATG in the cDNA insert is nucleotide 624 of Hcam3a (Fig. 2B) which corresponds to asparagin acid 150.

The plasmid Hcam1 (met141) contains an EcorI/XhoI fragment (nucleotides 505–1692, Fig. 1B) inserted into pBNY6n. The first ATG in the cDNA insert is nucleotide 505 of Hcam1 (Fig. 1B) which corresponds to asparagin acid 141. DNA sequences generated by PCR and new junctions were verified by DNA sequencing.

Yeast Strains—The genotypes of the yeast strains used in this work are shown in Table I. YKS42 was generated from SX50–1C by deleting

### Table I

| Strains       | Genotype | Source                  |
|---------------|----------|-------------------------|
| SX50–1C       | Matα his3 leu2 trp1 ura3 | Ivy et al. (27)          |
| YKS42         | Matα his3 leu2 trp1 ura3 Δpde1::HIS3 | This laboratory          |
| SX50–2D       | Matα his3 leu2 trp1 ura3 | J. Hicks                |
| YKS17         | Matα his3 leu2 trp1 ura3 Δpde2::TRP1 | This laboratory          |
| KXS15         | Diploid of YKS42 × YKS17 | This laboratory          |
| YKS44         | Matα his3 leu2 trp1 ura3 Δpde2::TRP1 | This laboratory          |
| YKS76         | Matα his3 leu2 trp1 ura3 Δpde2::TRP1 | This laboratory          |
| 5Bα           | Matα leu2 trp1 ura3 pep4-3 prb1-1122 prc1-126 | J. B. Hicks             |
| KXS24         | Diploid of YKS44 × 5Bα | This laboratory          |
| YKS76*        | Matα his3 leu2 trp1 ura3 Δpde1::HIS3 Δpde2::TRP1 pep4-3 prb1-1122 prc1-126 | (haploid segregant of KXS24) | This laboratory |

*YKS76 mates poorly with both Matα and Matα strains. It is assigned Matα because its growth is arrested near a Matα strain but not near a Matα strain. The presence of pep4-3, confirmed by DNA sequence analysis, rendered it difficult to determine the alleles at the prb1 and prc1 loci.

**TABLE I**

*Yeast strains*
the endogenous PDE1 gene (28) ( BamHI/HindI fragment containing the open reading frame deleted) and replacing it with the H153 gene (29) ( Eco471I/ BamHI fragment containing the open reading frame inserted). Standard methods were used for gene replacements (30) and yeast transformations (31). YKS17 was generated from SX50-2D by deleting the endogenous PDE2 gene (32) (nucleotides 7–1564 of PDE2 open reading frame deleted) and replacing it with the TRP1 gene (33) (1.4-kb EcoRI/HindIII fragment containing the open reading frame deleted). Standard methods were used for gene replacements (30) and yeast transformations (31).

The presence of the pep3-4 allele in YKS76 was confirmed by sequencing the region of the gene that contains the nonsense mutation present in this allele (34). Tests (35, 36) for the presence of the pep3-4 allele in 1122 and pcr1-126 alleles gave ambiguous results because pep3-4 is epistatic to their expression.

Biochemistry—YKS76 was transformed (37) with the plasmids Hcam1 (met141) and Hcam3a (met150) and the transformants were grown at 30°C in synthetic medium lacking leucine to 1–2 × 107 cells/ml, collected by centrifugation, washed once with water, and frozen in a dry ice-ethanol bath.

The yeast cell pellets were lysed and phosphodiesterase activity was assayed as described elsewhere (38) with the following modifications. [32P]cAMP and [32P]cGMP were used (25 Ci/mmol, ICN Biochemical). The [32P] product was separated from the substrate using charcoal (39, 40). Two volumes of 25 mg/ml activated charcoal in 0.1 M KH2PO4 were added to terminate the reaction. Following centrifugation, the supernatant was removed and quantitated by Cerenkov counting. Extracts were prepared from YKS76 containing only the pBNY6n vector yielded activity indistinguishable from assay background. Kinetic and inhibitor analyses were performed as described previously (38). Cyclic nucleotide hydrolysis was measured at substrate concentrations ranging from 0.03 to 100 μM. The specific activity of the substrate was held constant (0.2 Ci/mmol). The kinetic data were fitted to the Michaelis-Menton model using TableCurve 2D (Jandel Scientific). Inhibitor analyses were performed at 0.1 μM cGMP. At this substrate concentration the observed IC50 should approximate the apparent inhibitor constant (K,). The reaction time and amount of enzyme in the assay were adjusted to fit the double-reciprocal data to the Michaelis-Menton model using TableCurve 2D (Jandel Scientific). The IC50, as well as a parameter that determines the slope of the best-fit curve at the IC50, were obtained and the parameter affecting the slope is set to 1. The two-parameter logistic model was used when the highest concentration of inhibitor was unable to inhibit fully the enzymatic activity. All other data sets utilized the four-parameter model. The IC50 was set as a parameter that determines the slope of the best-fit curve at the IC50. The two-parameter model derives values for the minimum PDE activity, the maximum PDE activity, and the IC50 as well as a parameter that determines the slope of the fitted curve at the IC50. The four-parameter model derives values for the minimum PDE activity, the maximum PDE activity, and the IC50 as well as a parameter that determines the slope of the best-fit curve at the IC50. The two-parameter model derives values for the maximum PDE activity and the IC50. The minimum PDE activity is set as a parameter that determines the slope of the best-fit curve at the IC50. The four-parameter model was used when the highest concentration of inhibitor was unable to inhibit fully the enzymatic activity. All other data sets utilized the four-parameter model. Stocks of inhibitors were prepared in dimethyl sulfoxide (Aldrich), and the final solvent concentrations in the PDE assay never exceeded 2% (v/v). Vinpocetine was obtained from Asta Medica (New Haven, CT); vinblastine was a gift from Paul Feldman, Glaxo Inc. Research Institute (Research Triangle Park, NC); and 8-methoxymethyl IBMX was a gift from Jack Wells, Vanderbilt University (Nashville, TN). Cilostamide was synthet-
FIG. 2. A, map of Hcam3 cDNAs. The divergent 3'-ends of the cDNAs are represented by a hatched line (cDNAs He19a and He11a) or a jagged line (cDNA H3a). The two different open reading frames represented by these cDNAs are diagrammed. The portion of cDNA He19a represented as a dashed line has not been completely sequenced. B, nucleotide sequence and predicted protein sequence of Hcam3b (PDE1C1). The Hcam3a/b divergence follows nucleotide 2067. The D marked with a diamond is the first amino acid that is specific to the Hcam3 splice variant. cDNA H11a begins at nucleotide 611 and the protein expressed by the truncated Hcam3a(met150) construct begins at the methionine underlined at amino acid position 150. The positions of oligonucleotides used for RT-PCR are indicated above the sequence by numbered arrows. Nucleotide and amino acid residues are numbered on the right. C, nucleotide sequence and predicted protein sequences specific to Hcam3a (PDE1C3). The first nucleotide of this sequence corresponds to nucleotide 2067 of Panel B. Following this nucleotide, the two sequences diverge. The Hcam3a protein contains a glycine (G) rather than the aspartic acid (D) marked with a diamond in Panel B and extends another 79 amino acids before terminating. The cDNA insert from He19a contains a G at position 2246, whereas that from H11a contains an A. This change does not affect the amino acid sequence of the protein. Nucleotide and amino acid residues are numbered on the right.
Three phage were isolated from a human hippocampus cDNA library screened with a cDNA fragment derived from the bovine 61-kDa CaM PDE (17). DNA fragments derived from these phage were used to screen heart and aorta human cDNA libraries, and three additional phage were isolated. Subcloning and sequencing revealed that these six cDNAs correspond to two different genes, Hcam1 and Hcam3.

### RESULTS

**Hcam1—** Three Hcam1 cDNAs (H2a and H6a from hippocampus and A2d from aorta) provide a composite sequence of 2008 nucleotides (Fig. 1, A and B). This composite cDNA encodes a 535-amino acid protein with a predicted molecular mass of 61,251 Da that is similar to the bovine 61-kDa CaM PDE protein (5, 17) (Fig. 1, C). The three cDNAs used in building the composite (Fig. 1B) differ from it as follows (Fig. 1A). The composite sequence is identical to the sequence of cDNA H6a except that H6a is lacking nucleotides 625 and 627. These nucleotides are present in H2a and in the bovine 61-kDa CaM PDE. Deletion of them alters the reading frame. We believe their absence in H6a is a cloning artifact.

The cDNA insert in λH2a contains a 47-bp insertion following nucleotide 74 and diverges from the Hcam1 composite sequence following nucleotide 807 (Fig. 1A). The 47-bp insertion is in the 5′-untranslated region and does not alter the open reading frame of the cDNA. It contains sequences that show a good match to consensus splice donor and acceptor sequences (44), which suggests it may be an intron that was not spliced out of the mRNA from which the cDNA was made. It was not included in the Hcam1 composite sequence (Fig. 1B). The sequences following nucleotide 807, which show a good match to consensus splice donor sequences (44), may also represent an intron. It is known from an analysis of genomic DNA sequences that an intron is present at this position. These sequences were also not included in the composite sequence (Fig. 1B).

RT-PCR was used to isolate a portion of Hcam1 from human heart cDNA. A PCR-derived band extending from primer 2 to primer 3 (Fig. 1B) was sequenced. The 2 bp (626–627) were present, and the presumed intron following nucleotide 807 was absent, thus confirming our interpretation of the cDNA structures.

2. P. Snyder, unpublished observation.
the other samples. (The 2.4-kb/2.6-kb size difference was seen on two different blots (data not shown).) Lung and pancreas mRNA samples contain a small amount of the 2.6-kb mRNA. No hybridization signal was seen in the placenta sample. The transcripts were most abundant in mRNA from the brain, heart, kidney, and skeletal muscle.

We used RNase protection to determine if the specific mRNA structure predicted from the Hcam1 cDNAs was present in...
human RNA samples. The RNase protection probe extended across the two regions where the bovine and human sequences differed from each other (42-nucleotide insertion in human sequence and divergent 3′-ends, Fig. 4B). Using this probe a 688-nucleotide band was seen (Fig. 4A). This band was the size expected for protection of the entire Hcam1 probe. This confirmed that both the 42-nucleotide insertion and the human type of 3′-end were found in RNA from these tissues. A human mRNA lacking the 42-nucleotide insertion would have given rise to a 207–209-nucleotide band. No such band was detected. The smaller bands included a prominent 397-nucleotide band (Fig. 4A). This was the size expected for protection of the probe 5′ to the human/bovine divergence point and suggested the possibility that an additional form(s) of human Hcam1 mRNA might exist. Isolation of additional cDNAs will be required to test this suggestion.

Hcam1 mRNA expression was detected by RNase protection in RNA extracted from temporal cortex, hippocampus, brain stem, cerebellum, occipital cortex, heart ventricle, aorta, femoral and renal arteries, psoas skeletal muscle, and uterus (Fig. 4A). No expression was detected in the heart atrium, liver, or spleen.

Some of the RNA samples used in this RNase protection (indicated by stars in Fig. 4A) were partially degraded, possibly due to the length of time between donor death and tissue acquisition. The presence of protected bands using these samples indicated that the RNA included molecules that were long enough to span the RNase protection probe, although the signals obtained may be underrepresented or absent.

The results obtained in the RNase protections and the Northern blot agree reasonably well, although a few discrepancies were seen. The liver RNA used in the RNase protection showed no Hcam1 signal, while a different liver sample used in the Northern blot was positive. The skeletal muscle signal was also weaker in the RNase protection than might have been expected from the Northern blot results (again, a different sample). In the RNase protections, different samples of the heart and the testes gave qualitatively different results. Whether these differences reflect different expression levels in the different tissue donors, differing locations of the tissue within the organ, or some variation due to technical reasons is unknown.

Northern Analyses of Hcam3—For Hcam3 the cDNAs had predicted two distinct transcripts, one corresponding to Hcam3a and one corresponding to Hcam3b. Hybridization probes specific for each of these were prepared (see “Materials and Methods”) and used on Northern blots.

The Hcam3a-specific probe hybridized to a 5.6-kb mRNA present in the heart and brain samples (Fig. 5A). A weaker hybridization signal was detected in the lung, liver, kidney, and skeletal muscle samples. The Hcam3b-specific probe hybridized to an approximately 10-kb mRNA present in the brain and heart, and to a lesser extent in the lung mRNA (Fig. 5B). Additional faint bands were also detected with this probe. A Hcam3 catalytic region probe, which should hybridize to both transcripts, hybridized to both a 10- and a 5.6-kb transcript as well as additional smaller transcripts in the heart sample (data not shown). These results confirmed that both Hcam3a and Hcam3b represent transcripts that are present in human tissue mRNA. Hcam3 expression was also detected in the uterus and testes by RNase protection (data not shown).

Sequence Comparison of CaM PDEs—Human Hcam1, bovine 63-kDa CaM PDE (18), and human Hcam3 show 59% identity of amino acids when the gaps and the divergent carboxyl termini are excluded from the comparison (Fig. 6). Compared pairwise, Hcam1 and bovine 63-kDa CaM PDE are 65% identical as are bovine 63-kDa CaM PDE and Hcam3. Hcam1 and Hcam3 are 77% identical. A partial sequence for the human counterpart of the bovine 63-kDa CaM PDE has been reported (19), and it is 97% identical to the bovine sequence. Thus the greater identity of human Hcam1 and Hcam3 to each other than to the bovine 63-kDa CaM PDE is probably not a species difference. Hcam3 differs from Hcam1 and the 63-kDa CaM PDE by the presence of a 9-amino acid insertion in the putative calmodulin binding domain. Hcam3a and 3b are also larger than Hcam1 and the 63-kDa CaM PDE because of the longer carboxyl termini found in the Hcam3 proteins.

Biochemical Characterization of Hcam1 and Hcam3 Proteins Expressed in S. cerevisiae—The proteins encoded by Hcam1, Hcam3a, and Hcam3b were expressed in a strain of yeast that lacked endogenous PDE activity (YKS76). Expression of full-length proteins resulted in very low PDE activity that was not stimulated by 10 μM calmodulin (data not shown). This cannot be explained by the simple absence of enzyme, because full-length Hcam1, Hcam3b, and Hcam3a (with the Hcam3b amino terminus) proteins were detected in these yeast extracts by Western analysis (data not shown). This result is also not a simple consequence of the yeast expression system because full-length human Hcam2 and the bovine 63-kDa CaM PDE can be expressed in yeast29 and the activity of the expressed protein is stimulated by calcium and calmodulin. In contrast to the full-length proteins, amino-truncated proteins beginning at methionine 141 of Hcam1 (Fig. 1B) or methionine 150 of Hcam3a (Fig. 2B and C) gave measurable PDE activity and hydrolyzed both cAMP and cGMP (truncated Hcam3b was not expressed).

FIG. 5. Hcam3 Northern blot analysis. The positions of the RNA markers are shown on the left. A, Hcam3a-specific probe (nucleotides 2068–2686 of Fig. 2C). B, Hcam3b-specific probe (nucleotides 2062–2652 of Fig. 2B).
The effect of substrate concentration on the initial velocity of cyclic nucleotide hydrolysis was examined. Hcam1(met141) had a $K_m$ for cAMP 15-fold greater than its $K_m$ for cGMP (Fig. 7A, Table II). The specific activity or maximal rate of hydrolysis for cGMP and cAMP was determined for the yeast extract (nmol min$^{-1}$ mg protein$^{-1}$). This enzyme had a higher maximal rate of hydrolysis for cAMP than for cGMP, although an accurate estimate for cAMP was not obtained because of the high substrate concentrations required.

The kinetic parameters of Hcam3a(met150) differed significantly from those of Hcam1(met141) (Fig. 7B, Table II). Hcam3a(met150) had a $K_m$ of 0.6 $\mu$M for cGMP and 0.3 $\mu$M for cAMP, among the lowest ever reported for a calmodulin-dependent PDE (4). The sequence encoded by the Hcam3a cDNA begins at amino acid 146, from that position through amino acid 630 Hcam3a and Hcam3b are identical and are shown as one line of sequence. The unique regions of Hcam3a and Hcam3b are indicated separately. Amino acids are numbered on the right. The putative calmodulin-binding regions and the phosphodiesterase catalytic domains are indicated.

**DISCUSSION**

We report here the isolation and characterization of cDNAs encoding two human type I PDEs: Hcam1 and Hcam3. Hcam1 corresponds to the bovine 61-kDa CaM PDE (5, 17). The 535-amino acid human protein differs from the bovine protein in two regions (a 14-amino acid insertion and a divergent carboxyl terminus). RNase protection studies show that the nucleotide sequences predicted by the human cDNAs are present in RNA from human tissues, thus these differences are not likely to be cloning artifacts. The RNase protection studies also raised the possibility of an additional splice variant near the 3'-end of the open reading frame. The bovine 61-kDa CaM PDE is believed to undergo alternative splicing near the 5'-end of...
the open reading frame since the bovine 59-kDa CaM PDE differs from the 61-kDa CaM PDE at the amino terminus but is otherwise identical to it (6). Two different human mRNA transcripts were observed on Northern blots. It is not known whether these correspond to different splice variants. This question can be addressed by the isolation of additional cDNAs.

The second human type I PDE reported here is Hcam3. Two splice variants, which encode proteins that diverge from each other at the carboxyl terminus, were observed. Hcam3a is predicted to be 709 amino acids in length (80,759 Da), assuming it has the same 5′-end as Hcam3b (see above). Hcam3b is 634 amino acids in length (72,207 Da). The Hcam3b protein extends only 4 amino acids beyond the Hcam3a/3b divergence point before terminating, whereas the Hcam3a protein extends an additional 79 amino acids beyond the divergence point.

The carboxyl-terminal region specific to Hcam3a is very basic (43). Whether this unique carboxyl terminus of Hcam3a has any consequences for the enzyme’s catalytic activity, interaction with calmodulin, stability, or intracellular localization is not known. Some of these possibilities can be tested by comparing the properties of Hcam3a and Hcam3b when full-length, active proteins are expressed. Preliminary results indicated that the $K_m$ values for full-length Hcam3a and Hcam3b are similar to the data for the truncated Hcam3a reported here.4

A 19-amino acid region that bound calmodulin was identified in the bovine 61-kDa CaM PDE (amino acids 24–42) by peptide/calmodulin binding studies (5). This region is predicted to form an amphipathic $\alpha$-helix, a feature often found in calmodulin binding domains (48). The human Hcam1 protein also contains this region (Fig. 1C). However the human Hcam3 protein has a different amino acid sequence (6/19 amino acids differ) and contains a nine amino acid insertion (Fig. 6). This insertion is found at the same relative position as the alternative splicing that yields the bovine 61- and 59-kDa CaM PDEs. The Hcam3 protein, both with and without the 9-amino acid insertion, is predicted to form an amphipathic $\alpha$-helix, although the insertion alters the amino acids that would be included. Characterizing the binding of CaM to Hcam3 will help determine the effect of the amino acid insertion.

Two sites of phosphorylation by cAMP-dependent protein kinase A have been identified in the bovine 61-kDa CaM PDE

![Figure 7](image)

**TABLE II**

| Enzyme          | Exp. | cAMP $K_m$ | Specific activity | cGMP $K_m$ | Specific activity |
|-----------------|------|------------|------------------|------------|------------------|
|                 |      | (μM)       |                  | (μM)       |                  |
| Hcam1(met141)   | 1    | 40 (1)     | 3.0 (0.2)        | 0.31 (0.005) |
|                 | 2    | 53 (1)     | 3.6 (0.3)        | 0.376 (0.007) |
|                 | 3    | 27 (2)     | 3.4 (0.1)        | 0.446 (0.005) |
|                 | 4    | 71 (3)     | 3.9 (0.1)        | 0.695 (0.002) |
|                 | 5    | 73 (2)     | 4.1 (0.6)        | 0.66 (0.03)  |
|                 | 6    | 38 (1)     | 3.2 (0.4)        | 0.40 (0.01)  |
| Mean            | 51 (19) | 2.61 (0.05) | 3.75 (0.4) | 0.5 (0.2) |
| Hcam3a(met150)  | 1    | 0.31 (0.03) | 0.56 (0.06)    | 3.14 (0.06) |
|                 | 2    | 0.40 (0.06) | 0.46 (0.05)    | 2.34 (0.05) |
|                 | 3    | 0.29 (0.05) | 0.70 (0.1)     | 2.97 (0.09) |
| Mean            | 0.33 (0.06) | 2.3 (0.3)   | 0.57 (0.12) | 2.8 (0.4) |

a These data were derived from six independent yeast cultures grown from a single transformant expressing Hcam1 and three independent cultures grown from a single transformant expressing Hcam3. Experiment numbers correspond to particular cultures.

b $K_m$ values were determined as described under “Materials and Methods” and the legend for Fig. 7. The value in parentheses is the standard error. Specific activity is expressed as nmol substrate hydrolyzed min$^{-1}$ mg$^{-1}$ of yeast extract. The value in parentheses is the standard error.

c nmol substrate hydrolyzed min$^{-1}$ mg$^{-1}$ of yeast extract, measured in the yeast extract, accurate values for the specific activity or maximal rate of hydrolysis were not obtained for Hcam1(met141) and CAMP because of the high substrate concentrations required.

d The standard deviation of the mean is in parentheses.

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4 V. Florio, L. Uher, P. Snyder, K. Loughney, and K. Ferguson, unpublished observations.
in Hcam1 and Hcam3. It is not known whether either the serine, threonine, or any additional sites are phosphorylated in the human proteins.

Excluding gaps and divergent termini, Hcam1 and Hcam3 proteins are 77% identical, whereas Hcam1 and the bovine 61-kDa CaM PDE proteins are 94% identical. When one compares the DNA sequences over the corresponding regions of the cDNAs, they show 76 and 93% identity, respectively. Thus the analogous genes in two different mammals show higher levels of identity than do two different type I CaM PDEs in the same organism. Presumably, this reflects the conservation of both the regulatory interactions and the specific functions that these PDEs fill in mammalian cells.

Hcam1 expression was seen in brain, testes, and kidney samples. Lower levels of expression were seen in heart and uterus. The pattern of human Hcam1 expression resembled that of the bovine 59- and 61-kDa CaM PDEs (17). Hcam3 also showed a similar pattern of expression and was most readily detected in brain, heart, and testes (data not shown) but was also found in lung, uterus (data not shown), and kidney. Hcam1 and Hcam3 were coexpressed in human tissues. Whether this coexpression extends to the cellular level is unknown.

The biochemical characterization of Hcam1(met141) is consistent with its identification as the human counterpart of the bovine 61-kDa CaM PDE. The K_m value for cGMP (3.5 μM) that is much lower than the K_m value for cAMP (51 μM), and the response of Hcam1 to inhibitors, are in general agreement with the literature values for the bovine 61-kDa CaM PDE (4, 50, 51).

The biochemical properties of Hcam3a(met150) are distinct from those of Hcam1 and the bovine 63-kDa CaM PDE. Of these proteins, Hcam3a(met150) has the highest affinities for both cAMP and cGMP. A variety of CaM PDEs with high affinity for both cAMP and cGMP have been reported. A CaM PDE with a 1 μM K_m for cAMP and cGMP was detected in immature rat testis (12) and in the germ cells of male mice (15). A 68-70-kDa CaM PDE was partially purified from mouse testis (13). This enzyme had a K_m of 2 μM for both cAMP and cGMP. It cross-reacted with polyclonal rabbit antiserum against the bovine brain CaM PDE. In view of the size of the human Hcam3a protein (72 kDa), the presence of Hcam3 mRNA in the testis and the low K_m for both cAMP and cGMP, the response of Hcam3a(met150), one of the splice variants of the Hcam3 gene may correspond to the rat testis CaM PDE activity described. CaM PDEs with low K_m values for cAMP and cGMP have been reported in heart (8, 9, 14), pancreas (7), and in olfactory receptor neurons (10) and may also correspond to Hcam3 gene products. The mouse and rat counterparts of Hcam3 have been recently isolated and expression of the rat gene in olfactory neurons has been detected (52).

A 74-kDa bovine brain CaM PDE has also been identified (11). Although Hcam3 and this 74-kDa protein are similar in size and are both expressed in the brain, their kinetic properties differ. Thus the 74-kDa protein is not likely to be Hcam3 and may represent a type I PDE for which cDNAs have not yet been isolated.

Although Hcam1(met141) and Hcam3a(met150) have distinctive kinetic parameters, they show similar sensitivities to inhibitors. The IC_{50} values for IBMX, 8-methoxy-I BMX, rolipram, and cilostamide differed less than 2-fold among these CaM PDEs. The apparent affinity of Hcam1(met141) for zaprinast was about 3-fold lower than that observed for Hcam3a(met150). The largest difference seen was with vinpocetine where the IC_{50} value for Hcam1(met141) (8.1 μM) was 6-fold lower than that observed for Hcam3a(met150) (50 μM). The reported IC_{50} values for inhibition of CaM PDEs by vinpo-
calcine vary from 20 to 95 μM among enzymes isolated from bovine aorta, brain, coronary artery, kidney, and rabbit aorta (46, 47). This range of values may reflect the partial mixture of CaM PDEs present in each tissue. The inhibition of the truncated Hcam1 and Hcam3 PDEs, which do not contain calmodulin binding regions, is consistent with the hypothesis that vinpocetine is a catalytic region inhibitor (46).

In contrast to the full-length proteins, amino-truncated Hcam1 and Hcam3 were active when expressed in yeast. It has been reported that amino-truncation by proteolysis leads to activation of bovine type 1 PDEs (5, 50, 51). The kinetic properties of the proteolyzed bovine enzymes were similar to the calmodulin-activated full-length enzymes (50, 51). The amino termini of the proteolyzed bovine 61-kDa CaM PDEs (5) are similar in position to the amino terminus of Hcam1 (met141), suggesting that the recombinant truncated Hcam1 is activated as well. Hcam3a (met150) is truncated at the corresponding methionine and thus may also be activated.

The presence in tissues of proteins derived from more than one CaM PDE gene and the observation that different splice variants of the same gene can generate proteins that appear to be regulated differently has complicated the interpretation of the properties of the proteolyzed bovine proteins. Continued characterization of each gene and its products should aid in understanding the cellular roles of CaM PDEs.

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