Differential Expression of the 61 kDa and 63 kDa Calmodulin-Dependent Phosphodiesterases in the Mouse Brain

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Based on their relative abundance and regulation by Ca^{2+} and by phosphorylation *in vitro*, it is thought that the Ca^{2+} /calmodulin-dependent phosphodiesterases (CaM-PDEs) are important modulators of cyclic nucleotide function in the brain. Two of the most abundant CaM-PDEs in the brain are the 61 kDa and 63 kDa isozymes. In this study, the regional and cellular expression of mRNA encoding these two different isoforms in mouse brain has been determined by *in situ* hybridization. The 63 kDa CaM-PDE mRNA has a widespread but uneven distribution. Very strong hybridization signals are present in the caudate-putamen, nucleus accumbens, olfactory tubercle, and dentate gyrus of the hippocampus. Somewhat lesser amounts of 63 kDa CaM-PDE mRNA are present in the olfactory bulb and piriform cortex. Weaker but still easily discernible hybridization signals are seen in several layers of the cerebral cortex, CA1 and CA3 regions of the hippocampus, amygdaloid nuclear complex, thalamus, hypothalamus, midbrain, brainstem, cerebellum, and spinal cord. A weak hybridization signal was detected in the globus pallidus of the basal ganglia. In general, the distribution of the 63 kDa CaM-PDE is very similar to that of dopamine receptors, suggesting that it may modulate dopamine function.

In contrast, the 61 kDa CaM-PDE mRNA has a more limited and much different distribution, with the highest level of expression in the cerebral cortex and in the pyramidal cells of the hippocampus. A moderate hybridization signal was detected in the medial habenula and amygdaloid nuclear complex. In addition, small subsets of neurons in several other regions showed specific hybridization. Both PDE mRNAs appear to be localized exclusively in neuronal cell bodies. Their distinct distribution suggests important but different physiological roles for these two isozymes in the regional regulation of cyclic nucleotides in the CNS. Since these two isozymes are differentially phosphorylated by cAMP-dependent and Ca^{2+} /CaM-dependent protein kinases, the differential expression also provides a potential mechanism by which these PDEs can differentially regulate cAMP and cGMP in different brain areas. The high expression levels in specific

subsets of neurons also suggest that agents increasing Ca^{2+} in these neurons will increase the rate of cyclic nucleotide degradation.

[Key words: phosphodiesterase, cyclic nucleotide, calmodulin, striatum, *in situ* hybridization, localization]

The importance of cyclic nucleotides as second messengers in the CNS is well established. For example, cAMP and cGMP regulate gene transcription (Nagamine and Reich, 1985; Yamamoto et al., 1988), cytoskeleton function (Greengard, 1987), postsynaptic potential (Sarvey et al., 1989), action potential propagation (Costa et al., 1982), neurotransmitter biosynthesis (Edelman et al., 1981; Nestler and Greengard, 1983) and release (Kandel and Schwartz, 1982; Schoffelemeier et al., 1985), and many other neuronal functions. Intracellular concentrations of cyclic nucleotides are modulated both through synthesis by adenylyl and guanylyl cyclases and also degradation by phosphodiesterases (PDEs) (Bentley and Beavo, 1992). Alterations in PDE activity regulate both the steady state level and the rate by which these intracellular signals can be changed. For most excitable tissues, changes in cellular Ca^{2+} concentration also provide an important signal for cellular control, often interacting with cyclic nucleotides (Berridge, 1984; Kramer and Levitan, 1988). Several Ca^{2+} /calmodulin (CaM)-dependent enzymes, including CaM-dependent adenylyl cyclase, CaM-dependent protein kinase II, calcineurin, and the CaM-dependent PDEs (CaM-PDEs), have their highest concentrations in brain and are therefore thought to be especially important in neuronal function (Xia et al., 1991; Matsuoka et al., 1992; Silva et al., 1992; Takaishi et al., 1992). Recently, it has been shown that in isolated brain synaptosome preparations, CaM-PDE activity strongly modulates cGMP responses elicited by nitric oxide (Mayer et al., 1992).

A rather large family of CaM-PDE isozymes are expressed in mammals, all of which can be activated by CaM in the presence of calcium. At least five different members of this family have been described and of these, the 61 kDa, 63 kDa, and 75 kDa isozymes are known to be present in the brain (Bentley and Beavo, 1992). Recently, cDNAs encoding the 61 kDa and 63 kDa isoforms have been cloned from bovine, rat, and mouse (Bentley et al., 1992; Polli and Kincaid, 1992; Repaske et al., 1992; Sonnenburg et al., 1993). The 61 kDa and 63 kDa CaM-PDEs are encoded by two separate genes (Novack et al., 1989, 1991; Charbonneau et al., 1991; Sonnenburg et al., 1993). The 61 kDa and 63 kDa isoforms have similar CaM-binding affinities, although many of their other properties are quite different. For example, they differ in primary sequence, molecular weight, substrate kinetics, and regulation by phosphorylation/dephos-

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phorylation (Beavo and Reifsnyder, 1990; Conti et al., 1991). The 63 kDa isozyme is phosphorylated and regulated *in vitro* by CaM kinase II (Sharma and Wang, 1986). Conversely, the brain 61 kDa isozyme is phosphorylated and regulated *in vitro* by cAMP-dependent protein kinase (Sharma and Wang, 1985; Hashimoto et al., 1989). In both cases phosphorylation renders the enzyme less sensitive to activation by CaM. No nucleotide or peptide sequence information has been reported for the 75 kDa isozyme(s) although it appears to have different kinetic properties than the other two CaM-PDE isozymes (Shenolikar et al., 1985).

Some information on the cellular and subcellular distribution of CaM-PDE(s) in rat brain has been obtained by histochemical methods (Ariano and Adinolfi, 1977a,b; Greenberg et al., 1978; Ariano and Appleman, 1979; Ariano, 1983) and by immunocytochemical techniques (Kincaid et al., 1987; Ludvig et al., 1991). However, in none of these cases has it been entirely clear which isozyme(s) was being detected. For example, the histochemical techniques do not differentiate between isozymes, and it has not been certain whether or not the antibodies used in immunocytochemical studies would interact with more than the 61 kDa isozyme. Nevertheless, these studies generally suggested that CaM-PDE(s) appears to be present in most brain regions, but is highly enriched in neuronal populations and selectively present in the soma and dendrites of regional output neurons. The electron microscopic studies demonstrated high levels of what are probably CaM-PDEs in the postsynaptic region of specific classes of neurons (Ariano and Appleman, 1979; Ludvig et al., 1991). These data support other morphological evidence for a primary role of cyclic nucleotide action in postsynaptic events. In the development of the brain, CaM-PDE levels increase rapidly during periods of active synaptogenesis (Kincaid et al., 1992). Taken together, these studies suggest that CaM-PDEs function as an interface between Ca^{2+} and cyclic nucleotide second messenger systems in the postsynaptic integration of Ca^{2+} -mediated neuronal inputs. However, to date the role(s) of individual CaM-PDE isoforms in the CNS has not been elucidated.

The gross tissue distribution for 61 kDa and 63 kDa CaM-PDE mRNA has been examined in several bovine brain regions by Northern blot analysis and RNase protection assay (Bentley et al., 1992; Sonnenburg et al., 1993). These data showed that both the 61 kDa and 63 kDa isoforms are expressed widely, yet differentially throughout the bovine CNS; however, they did not provide information about the cell type specificity of these PDEs.

In order to determine cell-specific expression of distinct CaM-PDE isoforms and to understand further the physiological reasons for the existence of multiple CaM-PDE isoforms in the CNS, we have systematically determined the distribution of 61 kDa and 63 kDa CaM-PDE mRNA in adult mouse brain using *in situ* hybridization. Our data identify major sites of 61 kDa and 63 kDa isozyme expression and illustrate a markedly different regional and cellular distribution for the mRNA encoding these two isozymes.

Materials and Methods

Construction of templates. In order to test the specificity of the probes used in this experiment, a variety of antisense mRNAs corresponding to different domains of the genes have been used (Fig. 1). The probes used for detecting 63 kDa CaM-PDE mRNA were derived from the bovine brain cDNA clone p12.3a (Bentley et al., 1992) and are designated p11.6.C6, p10.28.A5, p3P-C, and p9PE-2 (Fig. 1). For the con-

struction of p11.6.C6, a 1.7 kilobase (kb) polymerase chain reaction (PCR) product including the entire open reading frame (ORF) of the bovine brain 63 kDa CaM-PDE (from bases 94–1735 of clone p12.3a) was subcloned into a pBluescript vector for sequencing and *in vitro* transcription. Clone p11.6.C6 contains 33 bases of 5' and 3' untranslated sequence in addition to the entire ORF. Similarly, p10.28.A5 was constructed by subcloning a PCR product from nucleotides 94–422 of clone p12.3a. This N-terminal domain corresponds to the 63 kDa CaM-PDE CaM-binding region. Clones p3P-C and p9PE-2 were constructed directly from restriction fragments of p12.3a. The construct p3P-C is a PstI fragment of p12.3a, corresponding roughly to the region between CaM-binding domain and catalytic domain (nucleotides 363–885 of the p12.3a sequence). The plasmid p9PE-2 is a PstI-EcoRI fragment containing part of the 63 kDa catalytic domain from nucleotides 886–1276 of p12.3a. Under the hybridization conditions used here, there is no cross-hybridization between the 61 and 63 kDa transcripts.

The riboprobe template for detecting 61 kDa CaM-PDE mRNA was constructed from a bovine brain cDNA, designated pCaMPDE-40 (Sonnenburg et al., 1993) and is shown in Figure 1B. A 550 base pair restriction endonuclease cleavage fragment corresponding to the conserved domain of the 61 kDa CaM-PDE was isolated from a 1% low-melting-point agarose/ $1 \times$ TAE gel and subcloned into the SmaI site of the pBluescript SK(–) plasmid. This plasmid, designated pCaMPDE-40-C, was used for *in vitro* transcription.

Preparation of riboprobes. Prior to *in vitro* transcription, these plasmids were linearized with appropriate restriction enzymes for antisense or sense probes. The *in vitro* transcription was carried out at 37°C for 2 hr in a 10 μ l reaction mixture containing a final concentration of 1 mM each of the nucleotides CTP, GTP, ATP, 10.5 μ M α - 35 S-UTP (New England Nuclear; > 1000 Ci/mmol); 40 U of RNasin (Promega); 2 mM spermidine; 10 mM dithiothreitol (DTT); 10 mM NaCl; 40 mM Tris-HCl, pH 7.5; 0.5 μ g of linearized template DNA; and 5 U of T3 or T7 RNA polymerase. The transcription reaction was followed by DNase digestion of the template for 30 min at 37°C (RQ1 DNase, Promega), phenol/chloroform extraction, and ethanol precipitation. In general, the specific activities obtained were between 10^8 and 10^9 cpm/ μ g. Probe size was reduced to ~100–150 bases by chemical alkaline hydrolysis at 60°C with sodium carbonate buffer (pH 10.4). The time of hydrolysis was calculated by the formula $t_{\min} = (L_o - L_f)/(kL_oL_f)$ where L_o = initial length, L_f = final length, and k = 0.11 kb/min.

Preparation of tissue sections. Female mice (6–8 weeks old) were decapitated, and the brains were rapidly isolated, frozen in OCT on dry ice, and stored at -70°C until use. Sections (8–10 μ m) were cut in a cryostat, thaw mounted on a polylysine-coated microscope slide, and stored at -70°C .

In situ hybridization. Procedures for *in situ* hybridization were performed essentially as described previously (Yan et al., 1990). Briefly, the sections were fixed in 4% (w/v) paraformaldehyde, pretreated with 0.2 N HCl, 2 mg/ml proteinase K, and 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8), followed by graded dehydration for 2 min each in 30%, 60%, 80%, 95%, and 100% ethanol. Before hybridization, each section was prehybridized with 50 ml of hybridization buffer in the absence of probe under parafilm coverslips in a humid chamber at 50°C for 2–6 hr. The prolonged prehybridization significantly reduced the background without affecting the specific hybridization signal intensity. The sections were then briefly rinsed in $2 \times$ saline-sodium citrate (SSC), dehydrated by ethanol, and air dried. When background was a problem, we added an excess amount of unlabeled thionyl-ribose competitor to the hybridization and prehybridization buffer. This unlabeled thionyl-ribose was synthesized using a pBluescript vector without an insert as template.

The data for 61 kDa and 63 kDa CaM-PDE mRNA expression shown in Figure 3 are taken from serial sections hybridized with the different isozyme specific riboprobes. However, direct comparisons between relative levels of isozyme expression should be made with great caution since it has not been established whether or not the two different bovine riboprobes hybridize equally well to the two mouse isozyme mRNAs or whether or not they are equally protected from the RNase by the mouse mRNA. However, direct comparisons between tissue regions for a given riboprobe within the same section or even between sections should be valid. The hybridization buffer contained 50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, $1 \times$ Denhardt's solution, 10 mM DTT, 1 mM EDTA, 1 mg/ml yeast tRNA, and 100 μ g/ml synthetic poly(A) RNA. Hybridization was performed in buffer containing ^{35}S -labeled antisense riboprobes (0.1 μ g/ml/kb) at 50°C for 16–20 hr in

a humid chamber under glass coverslips sealed by rubber cement. After the hybridization, sections were washed twice with $2\times$ SSC, 10 mM DTT for 30 min each at 55°C . In order to remove nonspecifically bound, single-stranded probe, the sections were treated with a solution containing 20 $\mu\text{g}/\text{ml}$ RNase A, 100 U/ml RNase T1, 0.5 M NaCl, 10 mM Tris-HCl (pH 8), and 1 mM EDTA for 30 min at 37°C . The sections were further washed in $2\times$ SSC, 50% formamide, 10 mM DTT for 30 min at 55°C , and in $0.1\times$ SSC, 10 mM DTT for 30 min at 55°C . The sections were dried by graded dehydration with 30% to 100% ethanol, and then either coated with NTB2 emulsion (Kodak) and exposed 1–3 weeks at 4°C or put directly on Cronex video imaging film. The emulsion coated slides were developed, fixed, stained through the emulsion with hematoxylin or hematoxylin/eosin, and mounted with Permount (Fisher). Each comparative set of slides were dipped in the same batch of emulsion and exposed for the same time.

Nonspecific hybridization was determined by parallel incubation of adjacent sections with α - ^{35}S -labeled sense riboprobes. In some experiments, competition was carried out by adding a 100-fold excess of unlabeled antisense probes to the prehybridization and hybridization mixture.

Digital imaging. The images in Figure 3 were taken with a Nikon microscope using dark-field illumination of the emulsion. They were captured using a Dage NC 65 video camera, Scion LG-3 frame grabber, and the Macintosh version of NIH IMAGE 1.45 software. Lamp voltage was adjusted so that very few pixels were saturated even in the areas of highest hybridization signal. The digital images were then transferred via the clipboard to Adobe Photoshop for alignment and then into Persuasion 2.1 (Aldus) for lettering and printing. The gray scale values were inverted (since they are dark-field images), and a background value of 16 gray scale units was subtracted from each pixel. The remaining 240 units were arbitrarily divided equally into six different ranges of 40 units each. In order to obtain the summary data in Table 1, each range was assigned a different color as follows. Highest light intensity or hybridization signal is red followed by orange, yellow, green, blue, and purple at 40 unit intervals. Values of $4+$ for red, $3+$ for orange, $2+$ for yellow-green, and $1+$ for blue-green were assigned. The blue color corresponds to hybridization near background levels. A purple color was seen for both the sense and antisense probes in areas not containing tissue. It should be noted that the relationship between the amount of hybridization signal and the gray scale value is not linear, particularly at the higher silver grain densities.

Common molecular biology reagents and DNA- or RNA-modifying enzymes were purchased from Stratagene, GIBCO, Bethesda Research Labs, or Promega. The *in vitro* transcription kit was from Stratagene. Radioactive α - ^{35}S -UTP and nonradioactive α -S-UTP were from Du Pont products. The X-OMAT-AR film and the NBT-2 emulsion, developer, and fixer were from Kodak. Cronex film was from Du Pont.

Results

Specificity and reproducibility. The DNA fragments used to construct templates for the *in vitro* transcription of the 61 and 63 kDa CaM-PDE riboprobes are shown in Figure 1. The specificity of hybridization observed for the riboprobes under these experimental conditions was verified by the following criteria. First, tissue sections were pretreated with 200 $\mu\text{g}/\text{ml}$ of RNase A and 200 U/ml ribonuclease T_1 before hybridization, as a control for nonspecific binding of the probes to molecules other than mRNA. No hybridization signal was seen under these conditions (data not shown) indicating that the riboprobe hybridized specifically with CaM-PDE mRNA. Second, corresponding sense probes showed little or no hybridization signal (Fig. 2). Third, hybridization signals were abolished in experiments carried out in the presence of a 100-fold excess of the same unlabeled antisense probe. However, hybridization of radiolabeled 63 kDa riboprobe in the presence of a 100-fold excess of non-radioactive 61 kDa riboprobe and vice versa did not change the hybridization pattern, as predicted by the calculated degree of mismatch in the hybridization conditions used here. In addition, the localization for the two PDEs differed, indicating that each antisense probe does not detect mRNA for the other isozyme.

Figure 1. Map of 63 kDa and 61 kDa CaM-PDE cDNA clones used to construct riboprobes. Riboprobes were transcribed *in vitro* using template DNA constructed from different restriction endonuclease cleavage fragments of cDNAs encoding either the 63 kDa (*A*) or 61 kDa (*B*) CaM-PDEs. The location of cleavage sites are designated by arrows. The cDNAs p11.6.C6 and p10.28.A5 were synthesized using the PCR technique (see Materials and Methods). All cDNAs were subcloned into the pBluescript SK(–) plasmid. The boxes and solid lines denote coding sequence and untranslated regions, respectively. The solid or hatched boxes designate sequences encoding either the CaM-binding domain or catalytic domain, respectively.

Finally, riboprobes derived from different regions of the same gene resulted in an identical labeling pattern and the distribution of hybridization signal was identical and roughly additive when a mixture of two probes was used.

The reproducibility and consistency of CaM-PDE specific hybridization was confirmed as follows. *In situ* hybridization experiments were carried out with serial sections at approximately 300 μm intervals in five separate mouse brains. Selected brain regions were examined in four other animals. In addition, three different types of section (coronal, sagittal, and horizontal) were examined. Representative sections of brain regions containing structures displaying a substantial hybridization signal with either the 61 kDa or the 63 kDa CaM-PDE probes are shown. In all cases, the labeling was consistent among the sections from each brain.

Identification and localization of 61 and 63 kDa CaM-PDE mRNA throughout the CNS. *In situ* hybridization with the 63 kDa CaM-PDE RNA probes revealed that the 63 kDa CaM-PDE mRNA was widely expressed throughout the brain, but is clearly concentrated in certain anatomically distinct regions. The 63 kDa CaM-PDE mRNA appeared to be highly concentrated in the caudate-putamen (neostriatum), the nucleus accumbens, the fundus striati, the olfactory tubercle and the dentate gyrus of the hippocampus (Figs. 2–5). Strong hybridization

Table 1. Regional expression of 61 kDa and 63 kDa CaM-PDEs in mouse brain

Brain region	63 kDa mRNA	61 kDa mRNA
Olfactory system		
Olfactory bulb	+++	ND
Anterior olfactory nuclei	+++	++
Olfactory amygdala	++	++
Piriform cortex	+++	+/-
Olfactory tubercle	++++	+/-
Cerebrum		
Cortex		
Cerebral cortex	++	++
Entorhinal	++	++
Endopiriform	++	++
Clastrum	++	++
Hippocampus		
Hippocampal subiculum	++	++
Dentate gyrus	++++	ND
CA1-CA4	+	+++
Basal ganglia		
Caudate-putamen	++++	sc
Nucleus accumbens	++++	+
Diencephalon		
Thalamus		
Paraventricular nuclei	++	+
Lateral geniculate	++	+
Reuniens thal nucleus	++	+
Others	++	ND
Hypothalamus		
Ventromedial	++	+
Dorsomedial	++	+
Lateral	++	+
Tuber cinereum	++	+
Paraventricular nuclei	++	+
Arcuate nucleus	++	+
Zona incerta	++	+
Suprachiasmatic nuclei	++	+/-
Preoptic areas	++	+/-
Mammillary body	++	ND
Interpeduncular nuclei	++	+/-
Epithalamus		
Medial habenular	ND	++
Lateral habenular	+/-	ND
Subthalamus	+	+
Islands of Calleja	+/-	++
Fundus striati	++++	+
Ventral pallidum	++	+/-
Globus pallidus	+	+
Entopeduncular nucleus	+	ND
Amygdala		
Basolateral	++	++
Central	++	++
Medial	++	++
Intercalated	+	+
Bed nucleus stria terminalis	++	++
Septum		
Lateral	++	+
Medial	++	+/-
Nuclear diagonal band	++	+
Triangular septal nucleus	++	ND

Table 1. Continued

Brain region	63 kDa mRNA	61 kDa mRNA
Midbrain		
Inferior colliculus	++	+/-
Superior colliculus	++	+/-
Ventral tegmentum	++	+
Substantia nigra, pars compact	++	+
Substantia nigra, pars reticulata	++	+
Dorsal raphe	++	+
Brainstem		
Pontine nuclei	+++	ND
Reticular nuclei	++	+/-
Spinal trigeminal nuclei	++	+
Ventral cochlear nuclei	++	++
Spinal cord	++	+/-
Cerebellum		
Purkinje cells	++	ND
Deep cerebellar nuclei	++	+/-

The hybridization signal intensities are taken from digitally scanned images similar to those shown in Figure 3 and detailed in Materials and Methods. +++, very strong (red); ++, strong (orange); +, moderate (yellow-green); +, weak (blue-green); +/-, background or slightly above (dark blue); ND, not detectable (purple); sc, scattered.

signals also were observed in the olfactory bulb, and the piriform cortex. Moderately strong hybridization signals were present over most other regions including the cerebral cortex, CA1 and CA3 regions of the hippocampal formation, amygdaloid nuclear complex, thalamus, hypothalamus, midbrain, brainstem (including pons and medulla), cerebellum, and spinal cord (Figs. 2–6). A weak hybridization signal was detected in the globus pallidus of the basal ganglia. Little or no hybridization signal was found in the habenula of the epithalamus, choroid plexus, and ependymal cells lining the ventricles.

In contrast to the 63 kDa CaM-PDE mRNA, distribution of the 61 kDa CaM-PDE mRNA was more limited. The highest levels of expression were observed in the pyramidal cells of the hippocampus, islands of Calleja, and cerebral cortex (Figs. 2–4). A moderate hybridization signal was detected in the medial habenular nucleus and amygdaloid nuclear complex. A relatively weak hybridization signal was present in many hypothalamic nuclei and a few areas in the thalamus, midbrain, and brainstem. Both 61 kDa and 63 kDa CaM-PDE mRNA appeared to be exclusively located in neuronal cell bodies but not in glial-like cells or fiber tracts (e.g., Fig. 5).

CaM-PDE localization in the olfactory system. Neither 61 kDa nor 63 kDa CaM-PDE mRNA was detected in the olfactory mucous membrane despite reports of CaM-PDE activity in these cells (Borisy et al., 1992). However, strong hybridization with 63 kDa CaM-PDE mRNA was observed in many areas of the central olfactory system. For example, an extremely strong hybridization signal was present in pyramidal cells of the olfactory tubercle (Fig. 2–3), strong to moderately strong hybridization signals were present in the olfactory bulb, anterior olfactory nucleus, piriform cortex, endopyriform nuclei, and olfactory amygdaloid nuclei. The 63 kDa CaM-PDE mRNA in the olfactory bulb was expressed in the nerve layer, in the glomerular layer, in the tufted cells of external plexiform layer (especially in external sublayer of middle tufted cells, in the mitral cell layer, and in the internal granular cells), but not in ependyma

Figure 2. Localization of 61 kDa and 63 kDa CaM-PDE mRNA in mouse brain. Sagittal sections of mouse brain were hybridized with α - 35 S-labeled antisense riboprobes directed to mRNA encoding either the 61 kDa CaM-PDE (top) or 63 kDa CaM-PDE (middle) and visualized using dark-field photomicroscopy (see Materials and Methods). At the bottom is shown the sense control for the 63 kDa CaM-PDE. The sense probe control for the 61 kDa CaM-PDE looked identical (data not shown). *Acb*, nucleus accumbens; *Am*, amygdaloid complex; *AO*, anterior olfactory nuclei; *BS*, brainstem; *Cb*, cerebellum; *CP*, caudate-putamen (striatum); *DG*, granule cell layer of the dentate gyrus; *HiP*, hippocampal pyramidal cell layer; *IC*, inferior colliculus; *ICj*, islands of Calleja; *OB*, olfactory bulb; *S*, subiculum; *SC*, superior colliculus; *SN*, substantia nigra; *Th*, thalamus; *Tu*, olfactory tubercle. Magnification, 2.5 $\times$; scale bar, 1 mm.

(Fig. 3). Significant amounts of 61 kDa CaM-PDE mRNA were detected in the anterior olfactory nucleus and in many olfactory amygdaloid nuclei.

CaM-PDE localization in the cerebral hemisphere (cerebral cortex, hippocampus, septal nuclei, and subcortical basal ganglia). Most of the neurons in the caudate-putamen and nucleus

accumbens were very strongly labeled with 63 kDa CaM-PDE mRNA probes, but few neurons were labeled with 61 kDa CaM-PDE probes (Figs. 2, 3, 5). The hybridization with mRNA of 63 kDa CaM-PDE expressed in the globus pallidus was much weaker than that in the caudate-putamen. The intensity of hybridization with riboprobe to the 61 kDa CaM-PDE in the

Sagittal section, 63 kDa CaM-PDE

Coronal sections, 61 & 63 kDa CaM-PDEs

Figure 4. Differential distribution of CaM-PDE transcripts in the neocortex and hippocampus. *A* and *B*, High-magnification dark-field photomicrographs of coronal neocortex sections hybridized with either the 61 kDa (*A*) or 63 kDa (*B*) CaM-PDE riboprobes. The roman numerals (*I–VI*) denote the six different layers of the cerebral cortex. *C* and *D*, Dark-field photomicrographs of horizontal hippocampal sections hybridized with either 61 kDa (*C*) or 63 kDa (*D*) CaM-PDE antisense riboprobes. *CA1*, *CA3*, and *CA4*, layers of the hippocampus; *DG*, dentate gyrus; *S*, subiculum. Magnification: *A* and *B*, 25 $\times$; *C* and *D*, 10 $\times$. Scale bars, 100 μ m.

globus pallidus was similar to that with the 63 kDa CaM-PDE riboprobe. Both 61 kDa and 63 kDa CaM-PDE mRNAs were moderately expressed in many regions of amygdaloid nuclear complex including the medial, basolateral, and central amygdaloid groups in addition to the olfactory amygdala. Moreover, high concentrations of 63 kDa CaM-PDE mRNA is expressed

in the olfactory tubercle but not in the islands of Calleja. Conversely, 61 kDa CaM-PDE mRNA is expressed in islands of Calleja but not in the olfactory tubercle.

Both 61 and 63 kDa CaM-PDE mRNA is expressed in the cerebral cortex. However, variations in localization were seen for each transcript (Fig. 4). The expression of 61 kDa CaM-

Figure 3. Digital imaging of mRNA encoding the 61 kDa and 63 kDa CaM-PDEs in mouse brain. *a*, Dark-field image of a sagittal section hybridized with 63 kDa CaM-PDE antisense riboprobe. The letters refer to the approximate location of the coronal sections shown in *b–f* below. *b–f*, Dark-field images of paired coronal sections hybridized with either 61 kDa CaM-PDE probe (left panel of each pair) or 63 kDa CaM-PDE probe (right panel of each pair) were digitally captured and mRNA levels were quantitated as described in Materials and Methods. *g* is a cross-section through the spinal cord. For all panels, the color pattern represents different gray scale values. White or light gray areas of high hybridization signal intensity are represented with red, followed by orange, yellow, and green in decreasing order of hybridization signal intensity. The background hybridization of either tissue sections or areas having no specimen with the sense riboprobe are rendered blue or purple, respectively. Details are described in the Materials and Methods. *Am*, amygdaloid complex; *BS*, brainstem; *CP*, caudate-putamen; *Cx*, cortex; *DG*, dentate gyrus; *Ent*, entorhinal cortex; *GL*, glomerular layer; *Hip*, hippocampus; *HTh*, hypothalamus; *IC*, inferior colliculus; *ICj*, islands of Calleja; *IGr*, internal granular layer; *MHb*, medial habenular nucleus; *Mi*, mitral cell layer; *Pir*, piriform cortex; *Pn*, pontine nuclei; *Purk*, Purkinje cell layer of the cerebellum; *SC*, superior colliculus; *Thal*, thalamus; *Tu*, olfactory tubercle.

Figure 5. Differential distribution of CaM-PDE transcripts in the caudate-putamen: dark-field (*A, B*) and bright-field (*C, D*) photomicrographs showing coronal sections of the caudate-putamen hybridized with either 61 kDa (*A, C*) or 63 kDa (*B, D*) CaM-PDE antisense riboprobes. Most but not all neurons express the 63 kDa CaM-PDE mRNA, while significantly fewer cells express the 61 kDa CaM-PDE mRNA. Neurons showing intense CaM-PDE mRNA hybridization are designated by arrows. Arrowheads point to neurons having little or no hybridization signal. Magnification: *A* and *B*, 25 $\times$; *C* and *D*, 250 $\times$. Scale bars: *A* and *B*, 100 μ m; *C* and *D*, 10 μ m.

PDE transcript is prominent in layers V and VI, whereas relatively less transcript was present in layers II–IV. The 63 kDa CaM-PDE mRNA was expressed more evenly in the neuronal cell bodies of layers II–VI. Layer V can be subdivided into Va, which is composed of medium-sized pyramidal perikarya underlying layer IV, and Vb, which is composed of relatively large pyramidal cells above layer VI (Paxinos, 1985). The 63 kDa CaM-PDE mRNA was expressed predominately in layer Va, whereas the 61 kDa CaM-PDE mRNA was located largely in Vb. Strong hybridization signals in the granular cells of the dentate gyrus and moderately strong hybridization signals in the pyramidal cells of the hippocampus were found for 63 kDa CaM-PDE mRNA (Fig. 4). In contrast, the hybridization signal for 61 kDa CaM-PDE mRNA was seen only in the pyramidal cells of hippocampus (from CA1–CA4), and not in the granular cells of the dentate gyrus.

CaM-PDE Localization in the diencephalon (epithalamus, thalamus, hypothalamus, and subthalamus). In the epithalamus there was no significant hybridization signal for 63 kDa CaM-PDE mRNA; however, specific hybridization for the 61 kDa CaM-PDE mRNA appeared in the medial habenular nucleus (Fig. 3). In the thalamus, 63 kDa CaM-PDE mRNA seems to

be moderately expressed in all thalamic nuclei (Figs. 2, 3), whereas 61 kDa CaM-PDE mRNA could not be detected in most nuclei of this region, with the exception of weak hybridization signals in postparaventricular thalamus lateral geniculate and reuniens thalamus (Table 1). In the hypothalamus, moderate hybridization signals for 63 kDa CaM-PDE mRNA were found over all the nuclei, but only a relatively weak hybridization signal for 61 kDa CaM-PDE message was seen in many nuclei including the arcuate, ventromedial, dorsomedial, posterior, and lateral hypothalamus nuclei. In the subthalamic nuclei, both 63 kDa and 61 kDa CaM-PDE isoform transcripts were expressed weakly.

CaM-PDE localization in the midbrain. In the midbrain, a moderately strong hybridization signal for 63 kDa CaM-PDE mRNA was found in all nuclei observed (Fig. 2, 3). A few areas of this region, including the substantia nigra, ventral tegmentum, dorsal raphe, and colliculi nuclei, showed relatively weak hybridization signals for the 61 kDa isozyme.

CaM-PDE localization in the brainstem (pons and medulla) and spinal cord. A moderately strong hybridization signal for 63 kDa CaM-PDE mRNA was distributed widely throughout the pons and medulla, whereas much weaker hybridization with

Figure 6. Localization of 61 kDa and 63 kDa CaM-PDE transcripts in the cerebellar Purkinje cell layer: dark-field (*A, B*) and bright-field (*C*) photomicrographs showing sagittal cerebellar sections hybridized with CaM-PDE antisense riboprobes. Sections were hybridized with antisense riboprobes corresponding to either the 61 kDa (*A*) or 63 kDa (*B, C*) CaM-PDE. Arrows point to clusters of grains surrounding the lightly stained Purkinje cell nuclei. *ML*, molecular layer; *GL*, granular layer; *Purk*, Purkinje cells. Magnification: *A* and *B*, 50 $\times$; *C*, 250 $\times$. Scale bars: *A* and *B*, 50 μ m; *C*, 10 μ m.

61 kDa CaM-PDE transcript was detected in the same regions (Figs. 2, 3). Similarly in the spinal cord, a moderate to strong hybridization signal for 63 kDa CaM-PDE mRNA was observed in the large motor neurons of the gray matter. In contrast to the 63 kDa CaM-PDE isozyme transcripts, the 61 kDa CaM-PDE mRNA was expressed very weakly in this region (Fig. 3).

CaM-PDE localization in the cerebellum. Using 63 kDa CaM-PDE riboprobes, moderate hybridization was located in the Purkinje cell layer and not in the molecular or granular layers of the cerebellum (Fig. 6). In addition, the deep cerebellar nuclei were moderately labeled (Fig. 3). In contrast to 63 kDa CaM-PDE, quite weak 61 kDa CaM-PDE hybridization signals were seen in a few deep cerebellar nuclei such as the medial, lateral, and interposed cerebellar nuclei.

Discussion

This study examines the distribution in mouse brain of mRNA encoding two structurally distinct CaM-PDE isozymes using the technique of *in situ* hybridization. The specificity of the riboprobes was verified by the observations that corresponding sense probes did not give significant hybridization signals, and that

an excess of cold antisense probe abolished the specific hybridization. In addition, the difference in regional expression observed for the two isozymes confirms the fact that the riboprobes were able to distinguish between the 61 and 63 kDa isozymes.

The immunocytochemical distribution of one or more CaM-PDE(s) in rat brain has been reported previously using antibodies against a purified CaM-PDE preparation thought to contain largely 61 kDa CaM-PDE (Kincaid et al., 1986, 1992). These investigators found prominent staining in the somata and dendrites of specific regional output neurons such as cerebellar Purkinje cells, and hippocampal and cortical pyramidal cells of the rat brain. In the present studies, although many Purkinje cells in the cerebellum were labeled by the 63 kDa CaM-PDE riboprobes, 61 kDa CaM-PDE mRNA was not detected. It seems possible that the previously reported immunocytochemical localization of 61 kDa CaM-PDE in cerebellar Purkinje cells might actually have been due to antibodies against the 63 kDa isoform present in the sera used since it is difficult to remove all traces of 63 kDa CaM-PDE from preparations of the 61 kDa isozyme used for immunization. It is also possible that another, unknown CaM-PDE having a common peptide epitope but different nu-

cleotide sequence could also be present in this region. Alternatively, a 61-kDa CaM-PDE mRNA could be expressed in rat cerebellar Purkinje cells but not in the mouse.

The expression pattern we observed for the 63-kDa CaM-PDE mRNA generally agrees with previously reported Northern blot analysis of mRNA from different bovine brain regions with bovine 63 kDa CaM-PDE cDNA as a probe (Bentley et al., 1992) and of mRNA from different rat brain regions with rat 63 kDa cDNA as a probe (Polli and Kincaid, 1992). However, the expression of 61 kDa CaM-PDE mRNA in mouse brain as determined by *in situ* hybridization analysis appears to be more restricted expression than indicated by previous Northern blot analysis of mRNA from different bovine brain regions. These latter studies showed abundant 61 kDa CaM-PDE mRNA widely distributed in bovine brain including cerebral cortex, basal ganglia, hippocampus, cerebellum, and medulla/spinal cord (Sonnenburg et al., 1993). However, little or no 61 kDa CaM-PDE mRNA was noted in most regions of the basal ganglia, cerebellum, or spinal cord. It is possible that the different distributions result from a species distinction between cow and mouse. For example, differences have been reported between gerbils and rats in the localization of binding sites of a second messenger (cAMP) and of the PDE inhibitor rolipram (Araki et al., 1992). Marked differences were especially noticed in the hippocampus, where cAMP and rolipram binding activities were very low in gerbils but high in rats, illustrating that regional differences in cyclic nucleotide and PDE expression can occur between species.

In other tissues high levels of specific PDE isozyme expression are associated with specific functions of the region. The best examples of this are in the retina, where high levels of a cGMP-specific PDE mediate part of the visual transduction cascade (Fung et al., 1980), and the adrenal cortex, where high levels of a cGMP-stimulated PDE mediate most of the effect of atrial natriuretic peptide on aldosterone production (MacFarland et al., 1991). The very high levels of 63 kDa CaM-PDE mRNA present in the striatum suggest an important function for this isozyme in this region. The distribution of 63 kDa CaM-PDE in this region closely mimics that of the dopamine receptors; in particular the D_1 and D_2 receptors are prominently expressed in the caudate-putamen, nucleus accumbens, and olfactory tubercle (Gerfen et al., 1990; Weiner et al., 1990, 1991; Surmeier et al., 1992). It therefore seems likely that the 63 kDa CaM-PDE may modulate one or more functions of dopamine in this region. For example, D_1 receptors are known to activate adenylyl cyclase and what is probably a D_1 -like receptor present in the striatum also may increase inositol triphosphate (IP_3) and Ca^{2+} levels (Mahan et al., 1990). D_2 receptors, which also are present in the striatum, are coupled to inhibition of adenylyl cyclase (Memo, 1990; Bunney et al., 1991). The colocalization of D_1 and D_2 receptors in the same regions with the 63 kDa CaM-PDE suggests that in those cells containing only D_1 -like receptors and the CaM-PDE, an increase in Ca^{2+} occurring, for example, as a result of IP_3 generation would attenuate the duration and magnitude of the dopamine-induced cAMP signal. However, in cells containing only D_2 receptors and the 63 kDa CaM-PDE, one might expect that an increase Ca^{2+} (e.g., via stimulation of the metabotropic glutamate receptor) would synergistically work with dopamine to lower cAMP by activation of the CaM-PDE along with D_2 receptor-mediated inhibition of adenylyl cyclase. More complex interactions can be envisioned in cells that might contain both receptors and the PDE. Whether or not all three

proteins are expressed in any of the same striatal neurons has not been demonstrated. However, it seems very likely that many neurons in the striatum express both 63 kDa CaM-PDE and at least one type of dopamine receptor since at least 90% of striatal neurons express 63 kDa CaM-PDE mRNA (e.g., Fig. 5) and about 50% of these neurons express D_1 and about 50% express D_2 receptor mRNA in rat brain (Weiner et al., 1990).

Since both the 61 kDa and 63 kDa CaM-PDEs also hydrolyze cGMP with high efficiency, analogous arguments can be made for agents that modulate Ca^{2+} , and cGMP. For example, any neurotransmitters that increase calcium in neurons containing high concentrations of CaM-PDE would be expected to diminish the magnitude and duration of the cGMP signal via activation of the PDE. A major modulator of cGMP in the CNS is nitric oxide (NO), which stimulates soluble guanylyl cyclase (Snyder, 1992). NO is formed by NO synthase in a Ca^{2+} /CaM-dependent manner. Since NO is freely diffusible, NO synthase located in adjacent cells can influence cGMP levels in neighboring neurons. This cellular separation provides a mechanism by which increased Ca^{2+} can selectively modulate both cGMP synthesis and degradation. Recent data obtained using synaptosomal preparations suggest a complex regulation of this type for NO, calcium, and cGMP in rat brain (Mayer et al., 1992).

The likely interactions of Ca^{2+} , CaM, cAMP, and cGMP are made even more complex by the fact that *in vitro* both of the CaM-PDEs are phosphorylated and decreased in their affinity for CaM by the phosphorylation (Sharma and Wang, 1985; Hashimoto et al., 1989). CaM kinase II phosphorylates and decreases CaM-dependent activation of the 63 kDa CaM-PDE but not the 61 kDa isozyme. Conversely, cAMP-dependent protein kinase phosphorylates the 61 kDa CaM-PDE isozyme, rendering it less sensitive to activation by Ca^{2+} /CaM. Therefore, the selective localization of these two different PDE isozymes in different regions also provides a potential mechanism by which region selective, longer-term modulation of cyclic nucleotide responses could be accomplished by activation of these kinases. Given the recent evidence for CaM kinase II involvement in synaptic plasticity (Hanson and Schulman, 1992), along with the location of high PDE levels in the postsynaptic densities (Ariano and Adinolfi, 1977a; Ariano and Appleman, 1979), it seems possible that at least part of the effects of CaM kinase II could be mediated via phosphorylation of the 63 kDa CaM-PDE. Similarly, phosphorylation of the 61 kDa CaM-PDE by cAMP-dependent protein kinase would be expected to amplify and prolong cAMP-mediated effects on synaptic strength and neuronal gene expression in regions where this PDE is highly expressed.

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