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Characterization of Particulate Cyclic Nucleotide Phosphodiesterases from Bovine Brain: Purification of a Distinct cGMP-Stimulated Isoenzyme

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ABSTRACT: In the absence of detergent, ≈80–85% of the total cGMP-stimulated phosphodiesterase (PDE) activity in bovine brain was associated with washed particulate fractions; ≈85–90% of the calmodulin-sensitive PDE was soluble. Particulate cGMP-stimulated PDE was higher in cerebral cortical gray matter than in other regions. Homogenization of the brain particulate fraction in 1% Lubrol increased cGMP-stimulated activity ≈100% and calmodulin-stimulated ≈400–500%. Although 1% Lubrol readily solubilized these PDE activities, ≈75% of the cAMP PDE activity (0.5 μM [^{3}H]cAMP) that was not affected by cGMP was not solubilized. This cAMP PDE activity was very sensitive to inhibition by Rolipram but not cilostamide. Thus, three different PDE types, i.e., cGMP stimulated, calmodulin sensitive, and Rolipram inhibited, are associated in different ways with crude bovine brain particulate fractions. After solubilization and purification by chromtography on cGMP–agarose, heparin–agarose, and Superose 6, the brain particulate cGMP-stimulated PDE cross-reacted with antibody raised against a cGMP-stimulated PDE purified from calf liver supernatant. The brain enzyme exhibited a slightly greater subunit M_r than did soluble forms from calf liver or bovine brain, as evidenced by protein staining or immunoblotting after polyacrylamide gel electrophoresis under denaturing conditions. Incubation of brain particulate and liver soluble cGMP-stimulated PDEs with V_8 protease produced several peptides of similar size, as well as at least two distinct fragments of ≈27 kDa from the brain and ≈23 kDa from the liver enzyme. After photolabeling in the presence of [^{32}P]cGMP and digestion with V_8 protease, [^{32}P]cGMP in each PDE was predominantly recovered with a peptide of ≈14 kDa. All of these observations are consistent with the existence of at least two discrete forms (isoenzymes) of cGMP-stimulated PDE.

Cyclic nucleotide phosphodiesterases (PDEs) constitute a complex group of enzymes, which are found in varying amounts and proportions in different mammalian cells and tissues (Beavo et al., 1982). At least six or seven distinct types or classes of PDEs can be distinguished on the basis of their subcellular localization, biochemical, pharmacological, immunological, and physical properties, and regulatory mechanisms. Representatives of several types (Beavo, 1988), i.e., Ca^{2+}- and calmodulin-sensitive PDE (Kincaid et al., 1984), cGMP-stimulated PDE (Martins et al., 1982; Yamamoto et al., 1983a; Miot et al., 1985; Pyne et al., 1986; Whalin et al., 1988), cGMP-inhibited PDE (Grant & Coleman, 1984; Harrison et al., 1986; Degerman et al., 1987), and rod outer segment PDE (Baehr et al., 1979), have been extensively

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purified. There is little immunological or structural evidence of extensive homology between PDEs of different classes. Little is known about the evolutionary relationships among the major PDE classes, possible similarities in amino acid sequences or posttranslational modifications and processing of these proteins, and nucleotide sequences and splicing mechanisms of their genes (Charbonneau et al., 1986). Each major type of PDE may represent a family of isoenzymes, and in fact, three or four putative isoenzymes of the Ca^{2+}- and calmodulin-sensitive PDE family have been described in bovine brain, heart, and lung (Sharma & Wang, 1985, 1986) and mouse testis (Rossi et al., 1988).

As reported here, at least three distinct types of PDE, i.e., cGMP-stimulated, Ca^{2+}- and calmodulin-sensitive, and Rolipram-inhibited PDEs, seem to be associated, via different forces, with washed particulate fractions from bovine brain. Consistent with reports of Beavo et al. (1971) and Whalin et al. (1988) the cGMP-stimulated PDE was concentrated in brain particulate fractions. The particulate cGMP-stimulated PDE from cerebral cortex purified after solubilization with the nonionic detergent Lubrol exhibited on SDS–PAGE[1] an M_r slightly greater than that of soluble cGMP-stimulated PDEs from bovine brain or calf liver. Data from photolabeling of the brain particulate and liver soluble cGMP-stimulated PDEs with [^{32}P]cGMP and digestion with V_8 protease support the idea that although there are some differences in the amino acid sequences of the two PDEs, the [^{32}P]cGMP may be associated with similar domains. Taken together, these findings support the existence of at least two distinct isoenzymes in the cGMP-stimulated PDE "family".

Experimental Procedures

Materials. [2,8-^{3}H]cAMP and [8,5′-^{3}H]cGMP purchased from New England Nuclear were purified by chromatography on cellulose (thin layer) with 0.5 M ammonium acetate/ethanol, 2:5 (v/v), and DEAE-Sephadex A-25 (columns, 0.5 × 3 cm). [^{32}P]cGMP (≈1000 Ci/mmol), [^{32}P]cAMP (≈75 Ci/mmol), and V8 protease (*Staphylococcus aureus*) were purchased from ICN, Inc., Irvine, CA; *Crotalus atrox* venom, cAMP, 5′-AMP, cGMP, 5′-GMP, bovine serum albumin, ovalbumin, PMSF, pepstatin A, β-mercaptoethanol, Freund's complete and incomplete adjuvants, and soybean trypsin inhibitor were from Sigma; C^8-$H_2N(CH_2)_2NH$-cAMP–agarose and N^6-H_2N-$(CH_2)_2$-cAMP–agarose were from P-L Biochemicals; goat anti-rabbit IgG conjugated with horseradish peroxidase, 4-chloro-1-naphthol, acrylamide, *N,N′*-diallyltartardiamide, *N,N′*-methylenebis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulfate, SDS, Coomassie brilliant blue R-250, amido black, gelatin, and Tween-20 were from Bio-Rad; leupeptin was from Boehringer Mannheim; Hepes was from Calbiochem-Behring; Tris, DTT, and prestained standard proteins were from Bethesda Research Laboratories; nitrocellulose membranes were from Schleicher & Schuell; EDTA and sodium azide were from Fisher; epoxy-activated Sepharose 6B and DEAE-Sephacel were from Pharmacia; heparin–agarose was from Pierce; isobutylmethylxanthine (IBMX) was from Aldrich. OPC 3689 (cilostamide) was generously supplied by Dr. H. Hidaka of Mie University and Otsuka Pharmaceuticals, dipyridamole by Boehringer Ingleheim, Rolipram by Berlex Laboratories (Cedar Knolls, NJ), Zaprinast by May and Baker, Ltd. (London, England), and purified bovine brain calmodulin by Dr. R. L. Kincaid of the National Institutes of Health.

Preparation and Solubilization of Particulate PDEs (Table I). Fresh calf brains from a local abattoir were transported to the laboratory on ice. After removal of pia matter, cerebral cortex was homogenized in two volumes (w/v) of ice-cold buffer I (50 mM Hepes, pH 7.5/1 mM EDTA/1 mM NaN_3/0.4 mM PMSF/1 μM pepstatin A/2 μM leupeptin/1 μg/mL soybean trypsin inhibitor/1 mM DTT) with a Polytron (Kinematica) (Table I). The homogenate was centrifuged (23000*g*, 60 min). Supernatant 1 was removed, and the pellet was washed twice by homogenization in buffer I containing 500 mM NaCl followed by centrifugation (23000*g*, 60 min), to yield washes 1 and 2. The final washed pellet (pellet 1) was homogenized (Polytron) or sonicated in buffer I with 1% Lubrol PX, incubated at 4 °C overnight, and centrifuged (23000*g*, 60 min) to yield supernatant 4 (solubilized particulate PDEs) and pellet 2. For some experiments, portions of supernatant 1 and pellet 2 were brought to 1% Lubrol before samples of fractions were assayed for PDE activity.

Purification of Solubilized Particulate cGMP-Stimulated PDE. Supernatant 4 from 300 g of cerebral cortex was applied to 200 mL of C^8-$H_2N(CH_2)_2NH$-cAMP–agarose (C^8-cAMP–agarose) previously equilibrated with buffer I containing 1% Lubrol PX. The gel was washed with two bed volumes of the same buffer. The flow-through fraction was applied to 50 mL of N^6-$H_2N(CH_2)_2$-cAMP–agarose (N^6-cAMP–agarose) previously equilibrated with buffer I containing 1% Lubrol PX. The N^6-cAMP–agarose was washed with two bed volumes of the same buffer. The flow-through fraction of N^6-cAMP–agarose was applied to 40 mL of cGMP–Sepharose prepared by the method of Martins et al. (1982). After being washed with 10 bed volumes of buffer I containing 500 mM NaCl and 1% Lubrol PX, the cGMP–Sepharose was warmed to 23 °C and washed with five bed volumes of buffer II (50 mM Hepes, pH 7.5/1 mM EDTA/1 mM NaN_3/0.4 mM PMSF/1 μM pepstatin A/1 mM DTT) containing 10% glycerol/0.1% Lubrol PX/125 mM NaCl/10 μM cAMP (Miot et al., 1985). The PDE was then eluted with three bed volumes of buffer II containing 10% glycerol/0.1% Lubrol PX/125 mM NaCl/10 mM cGMP and applied to 1 mL of heparin–agarose equilibrated with buffer II containing 10% glycerol, 0.1% Lubrol PX, and 125 mM NaCl. The flow-through fraction from heparin–agarose was applied to 1 mL of DEAE-Sephacel equilibrated with the same buffer. After being washed with 20 bed volumes of the same buffer, the PDE was eluted with 2.5 mL of buffer II containing 10% glycerol, 0.1% Lubrol PX, and 250 mM NaCl.

Phosphodiesterase Assay. Cyclic nucleotide phosphodiesterase activity was assayed as described (Yamamoto et al., 1983a) at 30 °C in a total volume of 0.3 mL containing 50 mM Hepes (pH 7.5), 8.3 mM $MgCl_2$, 0.1 mM EGTA, ovalbumin (0.1 mg/mL), and 0.5 μM [^{3}H]cAMP or [^{3}H]-cGMP (20 000–30 000 cpm) with and without 1 μM cGMP or 100 nM calmodulin and 0.5 mM $CaCl_2$, as indicated. Assays were terminated and ^{3}H-labeled nucleosides isolated for radioassay as described by Yamamoto et al. (1983a).

Production and Purification of Antibody. Male rabbits were injected subcutaneously with cGMP-stimulated PDE purified from the soluble fraction of bovine liver (Yamamoto et al., 1983a) in complete Freund's adjuvant and several weeks later with PDE in incomplete Freund's adjuvant. Two weeks after the second injection, rabbits were bled; the serum IgG

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; IBMX, isobutylmethylxanthine; PAGE, polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

fraction was prepared by chromatography on a column of staphylococcal protein A–Sepharose (Pharmacia). Anti-calf liver PDE IgG was affinity purified by adsorption to and elution from immobilized purified bovine brain particulate PDE according to the method of Olmsted (1986). Purified brain particulate PDE (10 μg) was subjected to SDS–PAGE (10% gels) (cf. below) and transferred to nitrocellulose. Strips containing the purified PDE ($M_r \approx 100\,000$) were cut from the nitrocellulose and incubated with anti-PDE IgG. Strips were washed with TTBS (TBS containing 0.05% Tween-20) and TBS (20 mM Tris, 50 mM NaCl, pH 7.5); antibody was eluted with 2 mL of 20 mM glycine hydrochloride buffer, pH 2.4, and neutralized with 1 M Tris-HCl, pH 8.0.

SDS–Polyacrylamide Slab Gel Electrophoresis and Immunoblot (Western Blots). Proteins were precipitated with 10% TCA and, after centrifugation, solubilized in 125 mM Tris base/10% glycerol/1% SDS/10% β-mercaptoethanol and 0.005% bromophenol blue. After being heated (2 min, 100 °C, Lab-Line multiblock heater No. 2093) samples were subjected to SDS–PAGE in 10% large gels (1.5 × 90 × 140 mm) with a (1.5 × 35 × 140 mm) stacking gel according to the method of Laemmli (1970). Mobility and M_r of the peptides (stained with amido black or Coomassie blue as indicated) were calculated from comparison with the mobilities of prestained standard proteins (myosin, M_r = 200 000; phosphorylase, M_r = 97 400; bovine serum albumin, M_r = 68 000; ovalbumin, M_r = 43 000; α-chymotrypsinogen, M_r = 25 700; β-lactoglobulin, M_r = 18 400; lysozyme, M_r = 14 300) from BRL Laboratories (Bethesda, MD). In some experiments proteins were transferred from polyacrylamide gels to nitrocellulose membranes (electrophoretic transfer at 100 V for 10 h, followed by 200 V for 1 h in 20% methanol/0.18 M glycine/0.025 M Tris/pH 8.3 in a Bio-Rad Trans Blot cell), and the blots were stained with amido black or incubated with antibody. Sections of nitrocellulose membranes were incubated at room temperature in TBS containing 3% gelatin and 3% bovine serum albumin and then washed twice with TTBS. After being washed, membranes were incubated (overnight, room temperature) with rabbit anti-bovine PDE IgG diluted in TTBS containing 1% gelatin. After being washed with TTBS, they were incubated (4 h, room temperature) with a second antibody (horseradish peroxidase conjugated goat anti-rabbit IgG) diluted in TTBS containing 1% gelatin. Peroxidase-conjugated antibody was detected with the chromogenic substrate 4-chloro-1-naphthol.

Photoaffinity Labeling. The photoaffinity-labeling reaction was carried out in 0.1 mL containing purified cGMP-stimulated PDE (usually ≈ 4 μg) in buffer II containing 250 mM NaCl, 10% glycerol, 0.1% Lubrol PX, 5 μg of ovalbumin, 0.032 μM [^{32}P]cGMP or 0.7 μM [^{32}P]cAMP, with or without 200 μM IBMX, and the indicated concentrations of unlabeled cAMP, cGMP, or 8-Br-cGMP. After incubation for 30 min in Microtest III flexible immunoassay plates on ice in the dark, samples were irradiated for 15 min with a Mineralight (254 nm) lamp (Ultraviolet Products, Inc.) 6 cm from the top of the plates. The reaction was terminated by the transfer of 0.09 mL of reaction mixture to 1 mL of cold 10% TCA. After centrifugation, the pellets were dissolved in 125 mM Tris base containing 10% glycerol, 1% SDS, 10% β-mercaptoethanol, and 0.005% bromophenol blue and subjected to SDS–PAGE as described above. After electrophoresis, the gel was stained for protein, dried, and subjected to autoradiography using Kodak XAR2 film and intensifying screens.

Proteolytic Digestion and Peptide Mapping (Cleveland et al., 1977). After TCA precipitation and solubilization, samples were subjected to SDS–PAGE in 10% minigels (0.75 × 90 × 50 mm). The gels were stained for 20 min in 0.1% Coomassie blue/10% acetic acid/25% isopropyl alcohol and destained with 5% methanol/10% acetic acid for 20 min. Bands corresponding to PDE were cut from the gels, trimmed, and soaked in 125 mM Tris, pH 6.8/0.1% SDS/1 mM EDTA for 30 min at room temperature with gentle swirling. The trimmed slices were then diced into pieces (≈1 × 1 mm) and applied to the bottoms of sample wells of a large (15%) polyacrylamide gel (1.5 × 70 × 140 mm) with a stacking gel (1.5 × 50 × 140 mm); the gel pieces were overlaid with 20 μL of soaking buffer containing 10% glycerol, 20 μL of soaking buffer containing V_8 protease (endoproteinase Glu-C, dissolved in 10% glycerol), and 30 μL of 125 mM Tris base/10% glycerol/1% SDS/10% β-mercaptoethanol/0.005% bromophenol blue. Electrophoresis was initiated (175 V), and the current was turned off when the bromophenol blue reached the top of the running gel. After 30 min, electrophoresis was continued; following electrophoresis, gels were stained with Coomassie blue R250, destained, dried, and subjected to autoradiography using Kodak XAR2 film and an intensifying screen. Mobility and M_r of peptides were calculated from comparison with those of standard proteins (phosphorylase, M_r = 97 400; bovine serum albumin, M_r = 68 000; ovalbumin, M_r = 43 000; carbonic anhydrase, M_r = 31 000; soybean trypsin inhibitor, M_r = 21 500; lysozyme, M_r = 14 000) from Bio-Rad (Richmond, CA).

Protein Determination. Protein content was assayed by use of Coomassie brilliant blue G-250 (Bio-Rad) with bovine serum albumin as the standard.

Results

As seen in Table I, after centrifugation (25000*g*, 60 min) of bovine brain homogenates prepared in the absence of detergent as outlined under Experimental Procedures, most of the calmodulin-stimulated cGMP PDE activity was recovered in the supernatant fraction (supernatant 1) whereas ≈80% of the cGMP-stimulated cAMP PDE activity was recovered in the washed particulate fraction (pellet 1). Only ≈5% of the total cGMP-stimulated cAMP PDE activity and ≈10% of the calmodulin-stimulated activity were recovered in the washes containing 500 mM NaCl (wash 1 and wash 2, Table I). Similarly, little activity was recovered in washes lacking NaCl or containing 5 mM EDTA (data not shown). cGMP-stimulated PDE activity was concentrated in particulate fractions from cerebral cortical gray matter or cerebellum (data not shown). In contrast, calmodulin-sensitive PDE activity was relatively high in soluble fractions from the cerebellum and white matter as well a gray matter (data not shown).

Brain particulate PDE activity was solubilized in buffer containing Lubrol PX. Washed pellet 1 was homogenized and sonicated in the presence of increasing concentrations of Lubrol and centrifuged. Portions of the total homogenate of pellet 1 and the supernatant after centrifugation were assayed to assess total activity in the presence of Lubrol as well as activity "solubilized" or released from pellet 1. Increasing Lubrol concentrations (1–5%) increased cGMP-stimulated PDE activity ≈100% and calmodulin-stimulated PDE activity ≈ 400–500% (data not shown and Table I). Addition of Lubrol to supernatant 1 produced a smaller increase in cGMP-stimulated and calmodulin-stimulated PDE activities (Table I). Most of the cGMP-stimulated and calmodulin-sensitive PDE activity in pellet 1 was solubilized by 1% Lubrol (data not shown and Table I). Although these results suggest that in pellet 1 more of the calmodulin-sensitive PDE than the cGMP-stimulated PDE was present in a "latent" or unexposed state, it is not possible to conclude that all effects of Lubrol

Table I: PDE Activity in Particulate and Supernatant Fractions from Bovine Brain[a]

fraction	Lubrol (1%)[b]	cAMP PDE [nmol min^{-1} (g of tissue)$^{-1}$][c] no cGMP	1 μM cGMP	Δ[d]	cGMP PDE [nmol min^{-1} (g of tissue)$^{-1}$][e] no CAM	CAM	Δ[d]	n
supernatant 1	0	2.3 ± 0.9	4.7 ± 1.4	2.4 ± 0.8	9.4 ± 1.9	33.2 ± 3.7	23.8 ± 2.7	7
	+	4.7 ± 0.3	7.6 ± 0.2	2.9 ± 0.2	20.3 ± 2.0	51.8 ± 3.3	31.5 ± 4.9	3
wash 1	0	0.8 ± 0.2	1.5 ± 0.6	0.7 ± 0.3	2.3 ± 0.4	6.7 ± 2.3	4.4 ± 1.3	6
wash 2	0	0.2 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.8 ± 0.1	1.7 ± 0.5	0.9 ± 0.5	6
washed particulate fraction								
pellet 1	0	21.1 ± 5.9	36.8 ± 6.7	15.7 ± 4.1	23.1 ± 5.6	27.2 ± 6.1	4.1 ± 0.8	5
	+	33.1 ± 10.1	59.1 ± 11.3	26.0 ± 3.9	51.1 ± 12.0	78.1 ± 17.2	27.1 ± 6.5	10
supernatant 4	+	11.1 ± 2.7	31.2 ± 5.0	20.1 ± 3.4	38.8 ± 9.2	57.6 ± 9.4	18.8 ± 3.5	9
pellet 2	+	24.9 ± 11.5	30.3 ± 12.5	5.4 ± 1.0	12.7 ± 1.6	18.3 ± 1.5	5.6 ± 1.2	4

[a] Bovine cerebral cortex was homogenized and fractionated as described under Experimental Procedures. Data are mean ± SD for the number of experiments in the last column (n). [b] Lubrol (final concentration 1%) was added to a portion of supernatant 1 and to a portion of homogenized pellet 1; centrifugation of a sample of the latter yielded supernatant 4 and pellet 2. [c] Hydrolysis of 0.5 μM [^{3}H]cAMP with or without 1 μM cGMP. [d] Increment in cyclic nucleotide hydrolysis produced by cGMP of CAM referred to elsewhere as cGMP-stimulated or calmodulin-sensitive PDE activity, respectively. [e] Hydrolysis of 0.5 μM [^{3}H]cGMP with or without 100 nM calmodulin and 0.5 mM $CaCl_2$ (CAM).

reflect solubilization or disruption of membrane structures, since, as seen in Table I, Lubrol seems to stimulate as well as solubilize enzyme activity.

Whereas most of the cGMP-stimulated cAMP and calmodulin-sensitive cGMP PDE activities were solubilized by Lubrol, ≈75% of the total cAMP hydrolyzing activity (with 0.5 μM [^{3}H]cAMP as substrate) not stimulated by cGMP remained associated with pellet 2 and was not released/solubilized in the presence of Lubrol (Table I). As shown in Figure 1E, cAMP PDE activity remaining in pellet 2 was very sensitive to inhibition by Rolipram (IC_{50} ≈0.5 μM) but not by cilostamide, a very potent inhibitor of the cGMP-inhibited low-K_m cAMP PDE from rat adipose tissue (Degerman et al., 1987), bovine cardiac tissue (Harrison et al., 1986), and human platelets (Grant & Coleman, 1984; Macphee et al., 1986). The solubilized cAMP PDE activity (assayed in the absence of cGMP) was relatively insensitive to either Rolipram or cilostamide (Figure 1B). The solubilized cGMP-stimulated cAMP PDE activity and the cGMP-stimulated PDE activity that remained associated with pellet 2 after Lubrol treatment were more sensitive to inhibition by dipyridamole and cilostamide than to inhibition by Rolipram (Figure 1C,F). Similar effects of dipyridamole and cilostamide on the cGMP-stimulated PDE purified from calf liver supernatant have been reported (Yamamoto et al. 1983b). cGMP PDE activity in supernatant 4 and pellet 2 was more sensitive to IBMX than to the other inhibitors tested (Figure 1A,D).

Lubrol-solubilized cGMP-stimulated PDE was purified from supernatant 4 as described under Experimental Procedures. Like the cGMP-stimulated PDE purified from calf liver (Yamamoto et al., 1983a) and bovine heart (Martins et al., 1982), the enzyme in supernatant 4 did not bind to either C^8- or N^6-cAMP–agarose at neutral pH, but was retained by cGMP–epoxy-activated Sepharose. After the column had been washed with buffer I containing 500 mM NaCl and 1% Lubrol and buffer II containing 125 mM NaCl/10% glycerol/0.1% Lubrol PX/10 μM cAMP (Miot et al., 1985), the cGMP-stimulated PDE was eluted with 10 mM cGMP. These preparations contained both PDE, a predominant protein band with $M_r \approx 100\,000$, and a variable amount of protein material with M_r in the same range as those of the myosin standards on SDS–polyacrylamide gel electrophoresis (Figure 2C). These and other higher molecular weight peptides were adsorbed almost completely by heparin–agarose (Figure 2C). The cGMP-stimulated PDE ($M_r \approx 100\,000$), which did not bind to heparin–agarose, was concentrated by adsorption to and elution from DEAE-Sephacel (Figure 2C); virtually all remaining contaminants could be separated from the cGMP-stimulated PDE by FPLC on Superose 6 (Figure 2A,B,D).

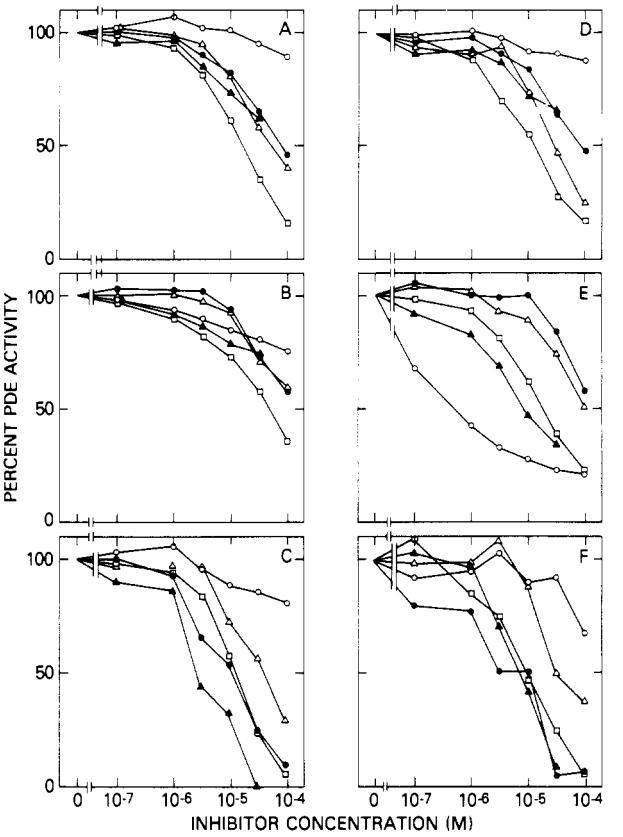


FIGURE 1: Effects of inhibitors on hydrolysis of [^{3}H]cAMP and [^{3}H]cGMP by supernatant 4 and pellet 2. Samples of supernatant 4 (A–C) and pellet 2 (D–F) were assayed without and with the indicated concentrations of IBMX (□), Zaprinast (△), dipyridamole (▲), cilostamide (●), and Rolipram (○). (Panels A and D) Hydrolysis of 0.5 μM [^{3}H]cGMP; (panels B and E) hydrolysis of 0.5 μM [^{3}H]cAMP; (panels C and F) increment in hydrolysis of 0.5 μM [^{3}H]cAMP produced by 1 μM cGMP. Activities are recorded relative to those in the absence of inhibitors = 100%.

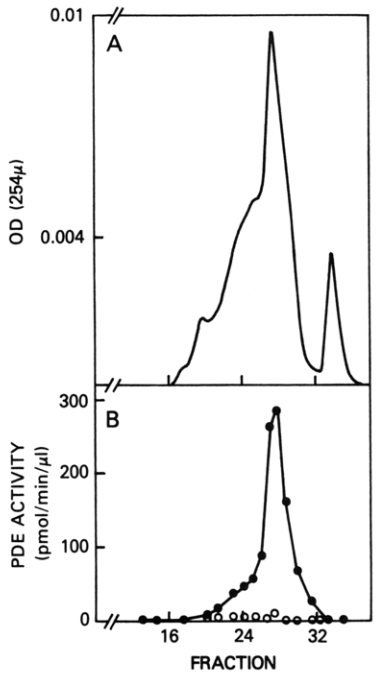
By use of this procedure, the particulate cGMP-stimulated PDE was purified 2000-fold from supernatant 4 solubilized from pellet 1 (Table II). The purified enzyme with 0.5 μM [^{3}H]cAMP as substrate was stimulated ≈10–15-fold by cGMP, with half-maximal stimulation at ≈0.4 μM cGMP; V_{max} (158 μmol min^{-1} mg^{-1}) and K_m^{app} (33 μM) for cAMP were also very similar to the values for the PDE purified from calf liver supernatants (Yamamoto et al., 1983a).

As seen in Figure 3, the purified particulate enzyme from bovine brain cerebral cortex exhibited an M_r slightly greater

Table II: Purification of cGMP-Stimulated PDE from Supernatant 4

	protein (mg)[a]	sp act. (nmol min^{-1} mg^{-1})[b]	activation (x-fold)[c]	yield (%)	purification (x-fold)
(1) supernatant 4	4900	1.3	4.2	100	1
(2) C^8-$NH_2(CH_2)_2NH$-cAMP–agarose; N^6-$NH_2(CH_2)_2NH$-cAMP–agarose	4900	1.1	4.4	89	
(3) cGMP–epoxy-activated Sepharose; heparin–agarose; DEAE–Sephacel	0.4	2668.3	13.0	16.7	2053

[a] Starting material, washed particulate fraction (pellet 1) from 300 g of cerebral cortex, was solubilized and purified as described under Experimental Procedures. Results reflect a representative preparation, repeated three times. [b] Based on increment in hydrolysis of 0.5 μM [^{3}H]cAMP produced by 1 μM cGMP. [c] Ratio of [^{3}H]cAMP hydrolysis in the absence of cGMP to that in the presence of 1 μM cGMP.



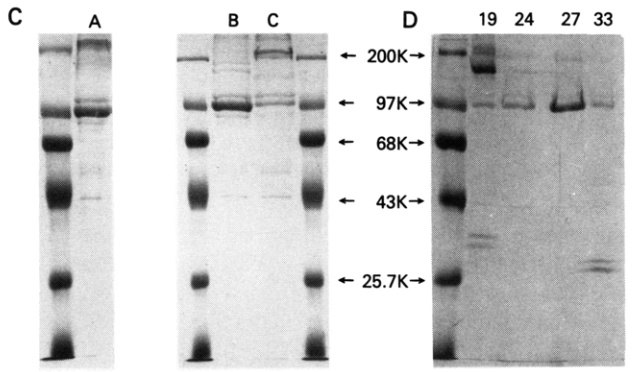


FIGURE 2: Chromatography of cGMP-stimulated PDE purified from supernatant 4 on Superose 6. cGMP-stimulated PDE was purified as described under Experimental Procedures and presented in Table II. (Panel C) SDS–PAGE of proteins (10 μg) eluted from cGMP–agarose (lane A), of cGMP-stimulated PDE and proteins (5 μg) that did not bind to heparin–agarose (lane B), and of proteins (5 μg) eluted from heparin–agarose with 1 M NaCl (lane C). (Panels A, B, and D) After concentration in a Centricon 30 (Amicon), enzyme (200 μg of protein that did not bind to heparin–agarose) was applied to Superose 6 (bed volume 25 mL) equilibrated and eluted with 50 mM Hepes (pH 7.5) containing 1 mM EDTA, 1 mM NaN_3, 1 mM DTT, 0.4 mM PMSF, 1 μM pepstatin A, 10% glycerol, 0.1% Lubrol PX, and 100 mM NaCl. Flow rate was 0.2 mL/min; fraction volumes were 0.5 mL. (A) Absorbance at 254 nm. (B) PDE activity assayed with 0.5 μM [^{3}H]cAMP in the absence (O) and in the presence (●) of 1 μM cGMP. (D) Fractions from Superose 6 (no. 19, 500 μL; no. 24, 50 μL; no. 27, 50 μL; no. 33, 500 μL) were precipitated with TCA, solubilized, and subjected to SDS gel electrophoresis as described under Experimental Procedures.

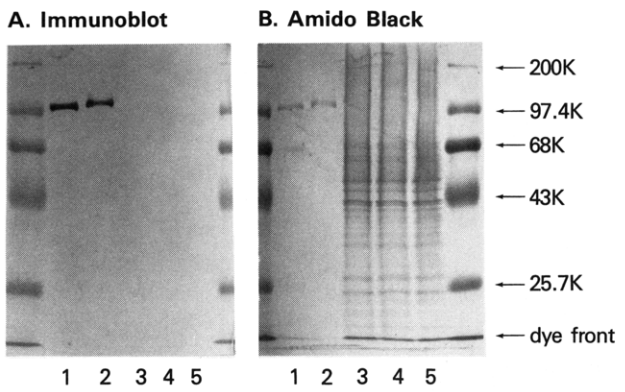


FIGURE 3: Western immunoblot of particulate and soluble cGMP-stimulated PDE. Purified calf liver supernatant cGMP-stimulated PDE (lane 1) and various fractions during purification of particulate cGMP-stimulated PDE from supernatant 4 (cf. Table II) (lanes 2–5) were precipitated with TCA and subjected to PAGE in SDS as described under Experimental Procedures. (Lane 1) cGMP-stimulated PDE (7 μg) purified from calf liver supernatant (plus added BSA); (lane 2) purified cerebral cortical cGMP-stimulated PDE (7 μg); (lane 3) cGMP–agarose flow-through fraction (30 μg); (lane 4) cAMP-agarose flow-through fraction (30 μg); (lane 5) supernatant 4 (30 μg). After transfer to nitrocellulose, one portion (A) was incubated with affinity-purified antibody against purified particulate cGMP-stimulated PDE, and the other (B) was stained with amido black as described under Experimental Procedures.

than that of the cGMP-stimulated PDE purified from calf liver supernatant (Yamamoto et al., 1983) or from bovine brain supernatant (by the procedure outlined under Experimental Procedures for the particulate PDE) (data not shown). During incubation of the purified particulate PDE with trypsin, immunoreactive peptides with M_r slightly less than the M_r of the untreated PDE ($M_r \approx 100\,000$) were generated (data not shown). It is not known if those immunoreactive peptides generated during incubation with trypsin are identical with the cGMP-stimulated PDE (with M_r lower than the brain particulate enzyme) in calf liver supernatant (Figure 3) or bovine brain supernatant 1 (Table I). In some preparations of the purified PDE some 200-kDa material was observed on SDS–PAGE; this material apparently cross-reacted with affinity-purified antibodies prepared by adsorption of anti-PDE IgG to and elution from purified particulate brain PDE antigen (≈100 kDa) immobilized on nitrocellulose strips after SDS–PAGE. In their studies, Whalin et al. (1988) reported that a monoclonal antibody raised against rabbit brain particulate cGMP-stimulated PDE cross-reacted with rabbit liver cytosolic cGMP-stimulated PDE.

As shown in Figure 4, the purified particulate cGMP-stimulated PDE could be photolabeled in the presence of 32 nM [^{32}P]cGMP; photolabeling did not occur without UV irradiation and was inhibited by 100 μM cGMP. Ovalbumin, which was present throughout the photolabeling procedure, served as an internal recovery marker and facilitated recovery after TCA precipitation. It did not apparently bind [^{32}P]cGMP and was not photolabeled. As shown in Figure 5, specific photolabeling with [^{32}P]cGMP was inhibited by much lower concentrations of cGMP (at least ≈100-fold) than of 8-Br-cGMP or cAMP. These findings are consistent with reports of other investigators, who demonstrated that cGMP-stimulated PDE

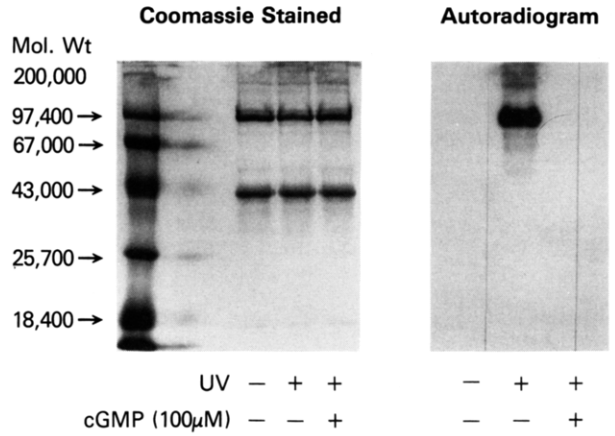


FIGURE 4: Photoaffinity labeling of the purified bovine brain cerebral cortical particulate cGMP-stimulated PDE. Purified particulate cGMP-stimulated PDE (4 μg) was incubated with buffer II containing 250 mM NaCl, 0.1% Lubrol PX, 5 μg of ovalbumin, 10% glycerol, 200 μM IBMX, and 0.032 μM [^{32}P]cGMP with or without 100 μM cGMP for 30 min on ice in the dark. As indicated, two of the samples were then irradiated for 15 min with a Mineralight UV lamp. 0.09 mL was withdrawn from all three samples and added to 1 mL of cold 10% TCA. After centrifugation, samples were dissolved and subjected to electrophoresis and autoradiography (40 h) as described under Experimental Procedures.

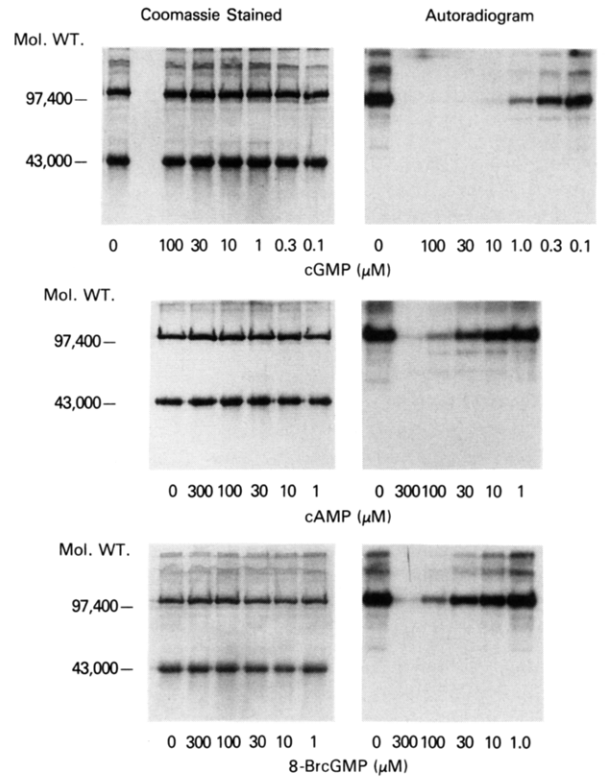


FIGURE 5: Effects of cGMP, 8-Br-cGMP, and cAMP on photoaffinity labeling of the purified bovine cerebral cortical particulate cGMP-stimulated PDE with [^{32}P]cGMP. Purified particulate cGMP-stimulated PDE (4 μg) was incubated as described under Experimental Procedures and in the legend of Figure 4 with 0.032 μM [^{32}P]cGMP in the absence or presence of the indicated concentrations of unlabeled cGMP, 8-Br-cGMP, and cAMP. Photoaffinity labeling, electrophoresis, and autoradiography (24 h) were carried out as described under Experimental Procedures and in Figure 4.

exhibited a higher affinity for cGMP than for cAMP and that cAMP and 8-Br-cGMP were much less potent than cGMP in activating the cGMP-stimulated PDE from rat or calf liver (Moss et al., 1977; Yamamoto et al., 1983a). Exposure of brain particulate and liver soluble PDEs to V_8 protease according to the procedure of Cleveland et al. (1977) resulted

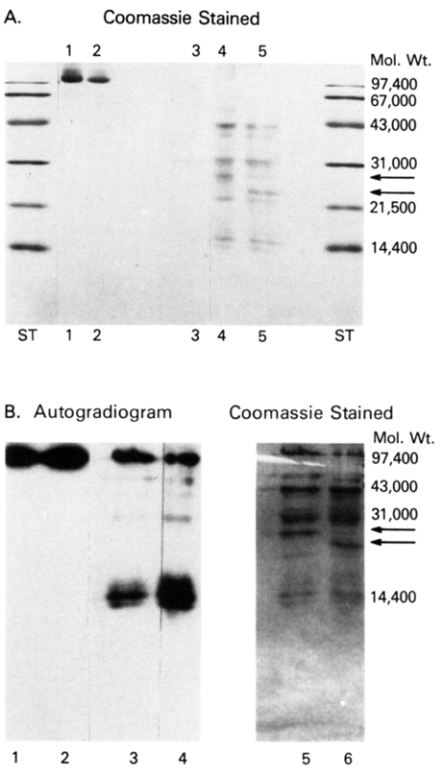


FIGURE 6: Cleveland proteolytic digestion and peptide mapping of purified cGMP-stimulated PDEs from bovine brain cerebral cortical particulate and calf liver supernatant fractions before and after photoaffinity labeling with [^{32}P]cGMP (Cleveland et al., 1977). (Upper panel) Purified cGMP-stimulated PDEs (≈4 μg applied to the initial SDS–PAGE) from brain (lane 1) and liver (lane 2) not incubated with V_8 protease prior to electrophoresis on the second gel; (lane 3) 1.0 μg of V_8 protease; cGMP-stimulated PDEs from brain (lane 4) and liver (lane 5) incubated as described under Experimental Procedures with 1 μg of V_8 prior to electrophoresis on the second PAGE. (Lower panel) Samples of purified brain particulate (5 μg) (lanes 1, 3, and 5) and liver soluble (5 μg) (lanes 2, 4, and 6) cGMP-stimulated PDEs were photolabeled with [^{32}P]cGMP in the presence of 200 μM IBMX prior to Cleveland protease digestion (0.2 μg of V_8 protease) and peptide mapping as described above and under Experimental Procedures. Autoradiographs were developed for 24 h.

in the production of several fragments of similar size (Figure 6). Distinct fragments of $M_r \approx 27\,000$ from the brain and ≈23 000 from the liver PDE were also produced (Figure 6). After photolabeling of the particulate and soluble PDEs with [^{32}P]cGMP and digestion with V_8 protease, the [^{32}P]cGMP was predominantly associated with ≈14-kDa peptides (Figure 6, lower panel). The peptides unique to the brain particulate and liver soluble forms were not labeled with [^{32}P]cGMP. The purified particulate PDE could be directly photolabeled with [^{32}P]cAMP (0.7 μM) as well as [^{32}P]cGMP (32 nM) in the absence or presence of IBMX (Figure 7). Peptide maps after photolabeling indicated that the same major peptides were apparently labeled with [^{32}P]cAMP or [^{32}P]cGMP.

Discussion

At least three distinct types of PDEs are associated, via different forces, with washed bovine brain particulate fractions. Of the total cGMP-stimulated PDE activity in bovine brain 80–85% is associated with particulate fractions from cerebral cortex gray matter. Very little of this activity was released in buffers containing 0 or 500 mM NaCl or 5 mM EDTA. These observations are very similar to those recently described for a cGMP-stimulated PDE purified from rabbit brain (Whalin et al., 1988). Addition of Lubrol PX increased bovine brain particulate cGMP-stimulated activity ≈100% but pro-

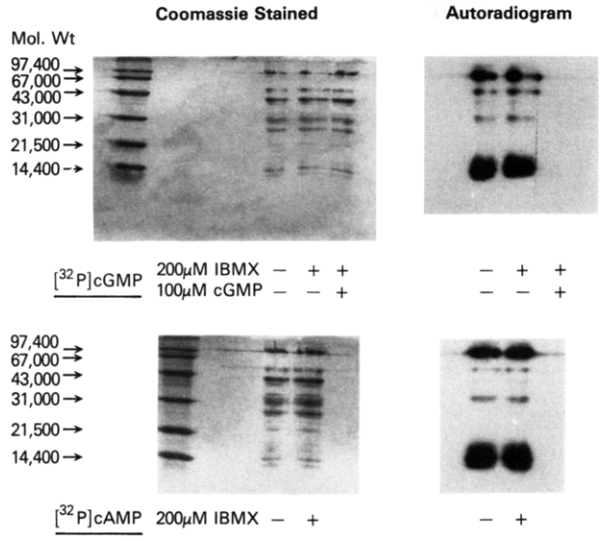


FIGURE 7: Peptide mapping of the bovine brain particulate cGMP-stimulated PDE after photolabeling with [^{32}P]cGMP or [^{32}P]cAMP. cGMP-stimulated PDE (≈5 μg/lane) was photolabeled as indicated with 32 nM [^{32}P]cGMP or 700 nM [^{32}P]cAMP in the absence or presence of cGMP or IBMX as indicated before protease digestion (0.2 μg of V_8 protease) and peptide mapping according to Cleveland et al. (1977) as described under Experimental Procedures. Autoradiographs were developed for 29 h.

duced a much larger increase in particulate calmodulin-sensitive PDE activity, suggesting that, relative to the cGMP-stimulated PDE, the particulate calmodulin-sensitive PDE is less accessible to substrate (i.e., "latent") or is more sensitive to activation by detergent.

In contrast to the calmodulin-sensitive and cGMP-stimulated PDEs, which are readily solubilized from particulate fractions with detergent, Rolipram-inhibited cAMP PDE activity remained in association with the particulate fraction in the presence of 1% Lubrol. Rolipram has been reported to potentiate the cAMP response to adenosine and histamine in brain slices, presumably by inhibition of cAMP PDE activity (Schwabe et al., 1976; Donaldson et al., 1988), and its therapeutic potential in depressive illnesses is being investigated (Wachtel, 1983a,b). Whether specific cerebral functions can be regulated by alterations in the activity of a particulate Rolipram-sensitive cAMP PDE remains to be seen. Earlier studies in 3T3-L1 adipocytes were consistent with the idea that Ro 20-1724 sensitive and Rolipram-sensitive cAMP PDE plays an important role in the process of 3T3-L1 adipocyte differentiation (Elks & Manganiello, 1985). In rat Sertoli cells, induction of a specific Ro 20-1724 sensitive cAMP PDE may be important in the regulation of cAMP in cells "desensitized" by exposure to follicle-stimulating hormone (Conti et al., 1986).

The so-called cGMP-stimulated cyclic nucleotide PDE has been purified from supernatant fractions of bovine cardiac, adrenal, and liver tissue (Martins et al., 1982; Yamamoto et al., 1983a; Miot et al., 1985), from cytosolic and particulate fractions of rat liver (Pyne et al., 1986), and from rabbit brain particulate fractions (Whalin et al., 1988). Our results indicate that, as has been reported for the calmodulin-sensitive PDE (Sharma & Wang, 1985, 1986), at least two distinct cGMP-stimulated forms can be isolated from bovine brain. One is soluble, and the other is found predominantly in association with particulate fractions from cerebral cortex. The particulate form, which accounts for ≈80–85% of total cGMP-stimulated PDE activity in bovine brain homogenates, exhibits a slightly greater subunit M_r than the soluble form from either bovine brain or calf liver. Exposure of the purified particulate PDE to trypsin results in accumulation of some immunoreactive peptides with M_r similar to that of the soluble forms. Whether this is fortuitous or has mechanistic implications for the appearance or distribution of the soluble form in intact cells is not known. Whalin et al. (1988) purified a cGMP-stimulated PDE from rabbit brain particulate fractions after solubilization with trypsin that exhibits physical and kinetic properties very similar to those reported for purified soluble forms. They suggested that the subunit M_r of the purified rabbit particulate PDE (after solubilization with trypsin) was identical with that of the soluble forms, although direct comparison of the soluble and particulate forms was not reported.

Exposure of brain particulate and liver soluble PDEs to V_8 protease resulted in the production of several fragments of similar size as well as peptides of different sizes, suggesting at least some differences in the amino acid sequences of the brain particulate and liver soluble PDEs. After photolabeling of particulate and soluble PDEs and digestion with V_8 protease, [^{32}P]cGMP was associated with peptides of similar size. Whereas the binding characteristics of the ^{32}P in the photolabeling experiments (i.e., binding of nanomolar cGMP in the presence of 200 μM IBMX and competition by low concentrations of cGMP but not cAMP or 8-Br-cGMP) would suggest that V_8 proteolytic mapping has allowed identification of large peptides containing "high-affinity" cGMP-binding sites, a major caveat related to photoaffinity labeling relates to the low stoichiometry of binding in these experiments. Peptides unique to the liver and brain forms were not apparently photolabeled. Taken together, these findings are consistent with the existence of at least two distinct GMP-stimulated PDE isoenzymes with perhaps similar or conserved as well as unique domains. In recent studies, Stroop et al. (1989) reported that, during photolabeling of the purified bovine heart cGMP-stimulated PDE with [^{32}P]cGMP, two putative cGMP-binding domains were labeled, one in a 36-kDa chymotryptic fragment and a second in a nonoverlapping 28-kDa cyanogen bromide fragment.

Recent immunocytochemical studies indicate that expression of the calmodulin-sensitive PDE is highly regulated in rat brain and that this PDE is concentrated in specific neuronal populations (Kincaid et al., 1987). It has also been suggested that calmodulin-sensitive PDE is associated with 10-nm filaments and microtubule preparations from bovine brain (Runge et al., 1979) and postsynaptic densities from canine brain (Grab et al., 1981) and that a cGMP-stimulated PDE is associated with coated vesicles from bovine brain (Silva et al., 1986). The specificity of these associations has not been established nor has the relative abundance of the different PDEs in the various neuronal populations. Little is known of the localization of the Rolipram-sensitive cAMP PDE. The presence of at least three PDEs in a crude bovine particulate fraction should permit us to determine whether these three distinct PDEs associate with the same oranelles/membranes via different forces or whether each PDE is located in different organelle/membrane systems. Distinct cellular distribution and/or subcellular localization of specific PDEs might very well be important in the regulation and intracellular compartmentalization of specific cAMP-mediated processes, e.g., in frog heart where the cGMP-stimulated PDE has been postulated to regulate a cAMP pool important for a slow inward Ca^{2+} current (Hartzell & Fischmeister, 1986).

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Registry No. PDE, 9040-59-9.

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