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Purification and partial characterization of membrane-associated type II (cGMP-activatable) cyclic nucleotide phosphodiesterase from rabbit brain

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Membrane-associated, Type II (cGMP-activatable) cyclic nucleotide phosphodiesterase (PDE) from rabbit brain, representing 75% of the total homogenate Type II PDE activity, was purified to apparent homogeneity. The enzyme was released from 13 000 × *g* particulate fractions by limited proteolysis with trypsin and fractionated using DE-52 anion-exchange, cGMP-Sepharose affinity and hydroxylapatite chromatographies. The enzyme showed 105 kDa subunits by SDS-PAGE and had a Stokes radius of 62.70 Å as determined by gel filtration chromatography. Hydrolysis of cAMP or cGMP showed positive cooperativity, with cAMP kinetic behavior linearized in the presence of 2 μM cGMP. Substrate concentrations required for half maximum velocity were 28 μM for cAMP and 16 μM for cGMP. Maximum velocities were approx. 160 μmol/min per mg for both nucleotides. The apparent K_{act} for cGMP stimulation of cAMP hydrolysis at 5 μM substrate was 0.35 μM and maximal stimulation (3–5-fold) was achieved with 2 μM cGMP. Cyclic nucleotide hydrolysis was not enhanced by calcium/calmodulin. The purified enzyme can be labeled by cAMP-dependent protein kinase as demonstrated by the incorporation of ^{32}P from [γ-^{32}P]ATP into the 105 kDa enzyme subunit. Initial experiments showed that phosphorylation of the enzyme did not significantly alter enzyme activity measured at 5 μM [^{3}H]cAMP in the absence or presence of 2 μM cGMP or at 40 μM [^{3}H]cGMP. Monoclonal antibodies produced against Type II PDE immunoprecipitate enzyme activity, 105 kDa protein and ^{32}P-labeled enzyme. The 105 kDa protein was also photoaffinity labeled with [^{32}P]cGMP. The purified Type II PDE described here is physicochemically very similar to the isozyme purified from the cytosolic fraction of several bovine tissues with the exception that it is predominantly a particulate enzyme. This difference may reflect an important regulatory mechanism governing the metabolism of cyclic nucleotides in the central nervous system.

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) diammonium salt; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, tosylphenylalanylchloromethylketone; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline (8 mM Na_2HPO_4/1.5 mM KH_2PO_4/2.7 mM KCl/137 mM NaCl); BSA, bovine serum albumin; Mes, 4-morpholineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; TMB, 20 mM Tris-HCl (pH 7.5)/5 mM magnesium chloride/5 mM 2-mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DAB, diaminobenzidine; Mab5C2-AG10, monoclonal antibody 5C2/Affi-gel 10 matrix; GAM-IgG, goat anti-mouse immunoglobulin serum; HRP, horseradish peroxidase; PDE, cyclic nucleotide phosphodiesterase.

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Introduction

The cyclic nucleotide phosphodiesterase enzyme system represents the enzymatic mechanism for the degradation of cyclic nucleotides and is a fundamental regulatory component in determining the intensity and duration of the cellular response elicited by hormones and neurotransmitters. Multiple forms of cyclic nucleotide phosphodiesterase with distinct physical, catalytic, immunological and regulatory properties are present in mammalian tissues [1]. One form with high specific activity (120–200 U/mg), termed Type II or cGMP-activatable cyclic nucleotide phosphodiesterase isozyme (EC 3.1.4.17) has been purified from cytosolic fractions of bovine heart [2], adrenal [2] and liver [3]. Purification of a low spec. act. (2–4 U/mg) Type II PDE from a particulate fraction of rat liver [4] has also been reported.

Purified bovine cytosolic Type II PDE isozymes hydrolyze both cAMP and cGMP with kinetic behaviors indicative of positive cooperativity [2,3]. The purified particulate rat liver Type II PDE reportedly shows positive cooperativity for cAMP only [4]. The rate of cAMP hydrolysis by Type II PDE is characteristically enhanced severalfold by low concentrations of cGMP [5,6]. Recently, the presence of distinct allosteric and catalytic sites for cGMP has been demonstrated using cyclic nucleotide analogs [7,8]. Binding studies using cardiac and adrenal Type II PDE demonstrate 1 mol of cGMP bound per mol of dimer [2] when performed in the presence of a competitive inhibitor.

The studies reported here were initiated to purify to apparent homogeneity, a membrane-associated Type II PDE for comparison of its properties to those of the purified, high specific activity cytosolic isozymes and the lower specific activity liver membrane Type II PDE. In addition to purification, we report the production of a monoclonal antibody to rabbit brain Type II PDE, the labeling of the enzyme by phosphorylation with cAMP-dependent protein kinase, and procedures for photoaffinity labeling of the enzyme with [^{32}P]cGMP. Portions of this work have been published in abstract form [9,10].

Materials and Methods

Materials

All chemicals were reagent grade or better. Frozen mature rabbit brains were purchased from Pelfreeze Biologicals and stored at −70 °C. [2,8-^{3}H]cAMP (spec. act. 36 Ci/mmol), [8-^{3}H]cGMP (spec. act. 19 Ci/mmol) and [^{32}P]cGMP (spec. act. 1123 Ci/mmol) were purchased from ICN. The tritiated cyclic nucleotides were purified by Dowex 1-X8 (200–400) chromatography before use [11]. Budget-solve scintillation fluid was from Research Products International. DE-52 (Whatman) was prepared according to manufacturers specifications and equilibrated in 20 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/5 mM 2-mercaptoethanol/20 mM benzamidine/0.02 mM TLCK/0.43 mM PMSF/115 U aprotinin (Buffer A) for enzyme purification. IBMX and Dowex 1-X8 (200–400) resin were from Aldrich Chemicals. The Dowex resin was treated with 0.5 M NaOH, 0.5 M HCl and distilled water washes prior to use. Epoxy-activated Sepharose 6B, Sephadex G-100 and Sepharose 4B were purchased from Pharmacia. Fast-flow hydroxylapatite (binding capacity of 12 mg BSA per g dry powder), fatty acid-poor BSA, and goat anti-mouse IgG antiserum were from Calbiochem. Agarose 0.5 M (200–400 mesh) and Affi-gel 10 were purchased from Bio-Rad Laboratories. Nitrocellulose paper (0.45 μm) was purchased from Sartorius. TPCK-treated trypsin was from Cooper Biochemicals, soybean trypsin inhibitor, TLCK, PMSF, cAMP, cGMP, ATP, DAB, benzamidine hydrochloride, dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine) and Ponceau S were from Sigma and aprotinin was from Boehringer Mannheim Biochemicals. Affinity-purified mouse IgG, GAM-IgG and HRP-linked GAM-IgG were from Zymed. The catalytic subunit of cAMP-dependent protein kinase purified from bovine heart [12] and [γ-^{32}P]ATP (spec. act. 140 Ci/mmol) were kindly donated by Dr. T.M. Lincoln and P3X63-A68.653 myeloma cells by Dr. J.G. Scammell. Papaverine (6,7-dimethoxy-1-(3′,4′-dimethoxybenzyl)isoquinoline hydrochloride) was obtained from Eli Lilly

and Co., milrinone (1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile) was from Sterling-Winthrop and rolipram (4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone) was from Pzizer Inc. SQ-65442 (1-ethyl-4-(ethylthio)-1*H*-pyrazolo (3,4-*b*)-pyridine-5-carboxylic acid ethyl ester) was given by Dr. Charles Free of E.R. Squibb and Sons Inc. and OPC-3689 (*N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butyramide) was a gift from Dr. H. Hidaka, Nagoya University School of Medicine, Nagoya, Japan.

Methods

Polyacrylamide slab gel electrophoresis

Polyacrylamide slab gel electrophoresis was performed with 7.5% separating and 5% stacking gels in 0.1% SDS according to Laemmli [13]. Native slab gel electrophoresis was performed at 4° C with 7% separating gels using the same procedure in the absence of SDS. For enzyme activity analysis, lanes from the native gel were cut into 1 mm slices and enzyme activity was determined using 40 μM [^{3}H]cGMP substrate (see below). Gels were stained for protein using Coomassie R-250 brilliant blue dye and destained with 45% methanol/10% acetic acid followed by 7.5% methanol/10% acetic acid. The gels were dried in Bio-Rad cellophane membrane backing for 2 h using a Bio-Rad Model 224 gel slab dryer and densitometric scans were obtained (E-C Apparatus Corp, model EC910).

Preparation of cGMP-Sepharose 6B affinity matrix. cGMP was covalently linked to epoxy-activated Sepharose for use as an affinity matrix using a modified procedure of Martins et al. [2]. A coupling density of 0.5 μmol cGMP per ml was estimated by spectrophotometric scan of the matrix suspended in 60% glycerol at 265 nm (pH 7.0) [2]. The cGMP-Sepharose affinity matrix was regenerated by washing with 2–3 bed volumes of 4 M guanidine hydrochloride 2 M NaCl, and 1 mM EDTA followed by 2 bed volumes of glass-distilled water. The matrix was incubated at room temperature for 2–4 h with 1 M ethanolamine (pH 8.0) and washed with 200 ml of 1 mM EDTA followed by 2 bed volumes of glass-distilled water. The affinity matrix was stored at 4° C in glass-distilled water.

Type II phosphodiesterase assay

PDE activities were determined by the modified two-step radioisotopic method of Thompson et al. [14]. Incubation mixtures (0.4 ml) contained 40 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 0.2 mM 2-mercaptoethanol, 30 μg fatty acid-poor BSA, 0.1–40 μM [^{3}H]cAMP or [^{3}H]cGMP (approx. 100000 cpm/assay), and enzyme giving less than 20% hydrolysis. Incubations were usually for 10 min at 30° C using 40 μM [^{3}H]cGMP substrate or 5 μM [^{3}H]cAMP substrate in the absence or presence of 2 μM cGMP. 1 unit of enzymatic activity is defined as the hydrolysis of 1 μmol of cyclic nucleotide per min. The initial velocity studies of the Type II PDE reaction were performed in the assay system described above with substrate concentrations ranging from 0.2 to 1000 μM. The initial linear reaction rates were used for velocity calculations. Assays with inhibitors were for 10 min at 30° C using 5 μM [^{3}H]cAMP substrate in the presence of 2 μM cGMP with varying concentrations of inhibitors. IC_{50} values were calculated from dose-response curves with 8–13 concentrations of inhibitor using the program Curvefit developed by P. Munson, D. Rodbard and M.L. Jaffe [15]. Papaverine was dissolved in glass-distilled water, dipyridimole in 100% methanol and other inhibitors in 100% DMSO. The vehicle concentration in the assays did not affect enzyme activity. Protein was determined by the Coomassie dye binding method of Bradford [16] with BSA as the standard.

Preparation of monoclonal antibody affinity matrix

5 ml of Affi-gel 10 matrix was prepared according to the manufactures specifications (Bio-Rad Technical Bulletin 1085) using isopropyl alcohol (3 ×) followed by cold deionized water (3 ×). The washed matrix was added to 13.5 ml of monoclonal antibody (Mab5C2, 23.4 mg/ml) previously dialyzed against 100 mM $NaHCO_3$ (pH 8.0). The mixture was incubated at 4° C for 4–6 h with gentle rotation. After incubation, the matrix was pelleted, resuspended in 1 M ethanolamine (pH 8.0) and incubated for 4 h to block residual active esters. The matrix was washed twice with 100 mM $NaHCO_3$ (pH 8.0) and was determined to have bound 19.4 mg IgG/ml of matrix.

Gel-filtration chromatography

Sephacryl S-200 (SF) gel filtration. This was performed in a 2.6 × 77 cm column at a flow rate of 0.9 ml/min using 5 mM Mes (pH 7.5)/1 mM dithiothreitol/50 mM NaF/500 mM sodium acetate/5% ethylene glycol/0.02 mM TLCK/15 mM benzamidine. 5 ml fractions were collected. The column was calibrated using standards of catalase (240 kDa), aldolase (156 kDa), BSA (67 kDA), ovalbumin (44 kDa) and cytochrome *c* (12.4 kDa). Thyroglobulin (669 kDa) was used to determine the void volume and Phenyl red was used to determine the included volume of the column. 99 μg purified enzyme was applied to the column and 40% of the applied activity was recovered. Fractions were assayed with 40 μM [^{3}H]cGMP substrate.

Sephadex G-100 gel filtration. This was with a 1.6 × 30 cm column at a flow rate of 0.32 ml/min using 20 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/5 mM 2-mercaptoethanol/15 mM benzamidine/500 mM sodium acetate with 3 ml fractions collected. 236 μg of purified enzyme was applied to the column and 85% of the applied activity was recovered using 40 μM [^{3}H]cGMP.

Agarose 0.5 M gel filtration. This was performed with a 1 × 111 cm column at a flow rate of 0.3 ml/min using 20 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/5 mM 2-mercaptoethanol/500 mM NaCl, with 2 ml fractions collected. The column was calibrated using standards of ferritin (443 kDa), catalase (240 kDa), γ-globulin (206 kDa), transferrin (81 kDa), ovalumin (44 kDa), and cytochrome *c* (12.4 kDa). 20 μg purified enzyme was applied to the column and 72% of the applied activity was recovered using 40 μM [^{3}H]cGMP substrate.

Sepharose 4B gel filtration. This was performed with a 2.6 × 70.5 cm. column at a flow rate of 0.96 ml/min using 40 mM Tris-HCl (pH 7.5)/10 mM magnesium acetate/5 mM 2-mercaptoethanol/200 mM NaCl/5% ethylene glycol. The column was standardized using standards of thyroglobulin (669 kDa), ferritin (443 kDa), catalase (240 kDa), β-amylase (200 kDa) and alcohol dehydrogenase (150 kDa), with 3 ml fractions collected. 60 μg purified enzyme was applied to the column and 80% of the applied activity was recovered using 40 μM [^{3}H]-cGMP substrate.

Cyclic AMP (5 μM) hydrolysis by peak fractions from all gel filtration columns was stimulated by 2 μM cGMP. The Stokes radius and diffusion coefficient of the Type II PDE were estimated by plots of the known Stokes radii and diffusion coefficients of the standards used versus their V_e/V_0 ratios.

Covalent labeling of phosphodiesterase by phosphorylation

1–5 μg purified Type II PDE was incubated with [^{32}P]ATP (10 μM; 2×10^6 cpm) and 10–200 U of purified catalytic subunit of cAMP-dependent protein kinase in a buffer consisting of 40 mM Tris-HCl (pH 7.5)/10 mM magnesium acetate/0.2 mM 2-mercaptoethanol. Incubations were for 30 min (30 °C) and were terminated by the addition of boiling SDS buffer (62.5 mM Tris-HCl (pH 6.8)/1% SDS/2 mM dithiothreitol/10% glycerol/0.05% Bromophenol blue) followed by additional boiling for 2 min. Samples were analyzed using 7.5% SDS-PAGE. ^{32}P incorporation was determined by autoradiography of the stained and dried gels using Kodak X-Omat AR X-Ray film in cassettes equipped with intensifying screens (6–12 h, at −70 °C).

Photoaffinity labeling of phosphodiesterase

1–5 μg purified Type II PDE from step 5 of purification procedure (see Results) was incubated for 5 min on ice in 40 mM Mes (pH 6.0) or 40 mM Tris-HCl (pH 9.0), 1 mM EDTA, and [^{32}P]cGMP (80–200 nM; (19–48) × 10^6 cpm) in the absence or presence of 1 mM cGMP. The enzyme was irradiated at a distance of 10 cm at 254 nm for 10 min. After photolysis, the incorporation of [^{32}P]cGMP into Type II PDE was determined by 7.5% SDS-PAGE and autoradiography of the stained and dried gels as described above. Type II PDE irradiated under the same conditions without [^{32}P]cGMP showed no difference in electrophoretic mobility, staining intensity or specific activity compared to enzyme which had not been irradiated.

Enzyme-linked immunosorbent assay (ELISA)

Hybridoma culture supernatants were screened for antibody production by ELISA, using purified Type II PDE as the antigen. Briefly, 0.2–0.5 μg of purified enzyme was bound to 96 well microtitre plates with 1% polylysine and 0.5% gluteralde-

hyde. The wells were washed (0.3 ml/enzyme assay with PBS and blocked with a 1% BSA/75 mM glycine solution followed by two additional PBS washes. Cultured cell supernatants (0.05 ml) were added and plates were incubated at 37°C for 30 min, washed with PBS, 3% BSA/1% Tween-20 solution followed by another PBS wash. Affinity-purified horseradish peroxidase-linked GAM-IgG in 0.1% BSA was added (0.05 ml) to each test well and incubated for 30 min at 37°C. After washes with PBS and 3% BSA/1% Tween-20, 0.05 ml ABTS/0.015% H_2O_2 was added to each test well. The development of a green color indicated positive antibody production and could be compared semiquantitatively with affinity-purified mouse IgG standards (0.1–5.0 μg) and media blanks.

Production of a phosphodiesterase monoclonal antibody

Monoclonal antibodies were produced against the Type II PDE using slight modifications of the methods of Köhler and Milstein [17]. A Balb/*c* mouse was immunized intraperitoneally with 30 μg purified Type II PDE mixed 1 : 2 with Freund's complete adjuvant. After 16 days, the mouse was boosted intraperitoneally with 10 μg purified PDE in saline. The mouse was killed 3 days later by CO_2 asphyxiation, the spleen was removed and spleen cells were fused with P3X63-A68.653 myeloma cells using 40% poly(ethylene glycol). Hybridomas were isolated by growth in selective hypoxanthine/aminopterin/thymidine medium. 14 colonies of Type II PDE antibody producing hybridomas were originally identified from 133 colonies (in 768 wells) by antigen ELISA (see above). Of these 14 strong ELISA positive growing colonies, one (labeled 5C2) quantitatively immunoprecipitated Type II PDE activity. The hybridoma, 5C2, was cloned by limiting dilution. The anti-Type II PDE antibody secreting hybridoma or cloned cells were grown in culture and ELISA-positive antibody obtained from the culture medium by 40% ammonium sulfate precipitation. Precipitated antibody was suspended in PBS, dialyzed for 12 h at 4°C against 200 volumes 10 mM Tris-HCl (pH 7.5) and stored at −20°C.

Immunoprecipitation of Type II PDE activity

Immunoprecipitation of enzyme activity. Type II PDE and various amounts of antibody were incubated for 30 min at 30°C using the same conditions as for PDE activity analysis (without substrates) in a final volume of 0.5 ml. The mixture was placed in a ice bath and the PDE-antibody complex precipitated using 10 μg affinity-purified mouse IgG and 0.05 ml GAM-IgG antiserum. After 25 min, the mixture was diluted to 1.0 ml with 40 mM Tris-HCl (pH 7.5)/10 mM magnesium acetate/0.2 mM 2-mercaptoethanol centrifuged for 10 min at 12000 × *g* in a Beckman Microfuge 12, and Type II PDE activity of the resultant supernatant and pellet was determined. The immunopellets were suspended using a Kontes teflon pestle. Affinity-purified mouse IgG and GAM-IgG antiserum had no direct effect on Type II PDE activity and showed no nonspecific precipitation of Type II PDE activity under these conditions.

Immunoprecipitation of enzyme protein. 5 μg of purified Type II PDE was incubated (12 h at 4°C with gentle rotation) with an excess Mab5C2-AG10 in a buffer consisting of 40 mM Tris-HCl (pH 7.5)/10 mM magnesium acetate/30 μg BSA in a total volume of 0.5 ml. After incubation, the matrix was pelleted by centrifugation in a Beckman Microfuge 12 at 12000 × *g* for 10 min, the supernatant was removed and the immunopellet was washed three times with PBS. Following washing, the immunopellet was suspended in 0.1 ml SDS sample buffer and boiled for 5 min. The mixture was centrifuged again at 12000 × *g* for 10 min and the resulting supernatant was subjected to 7.5% SDS-PAGE.

Immunoprecipitation of ^{32}P-labeled enzyme. 1 μg of purified Type II PDE was labeled with ^{32}P as described above. After 30 min, 0.1 ml of the Mab5C2-AG10 was added to the solution and incubated 12 h at 4°C with gentle rotation. In these experiments, the phosphorylation reaction was not terminated by boiling in SDS. After incubation, the matrix was pelleted by centrifugation, the supernatant was removed and the immunopellet was washed three times with PBS. The immunopellet was treated as described above and the supernatant was subjected to 7.5% SDS-PAGE. After electrophoresis, the stained and dried gels were subjected to autoradiography as described above.

Western blot transfer and immunoblot analysis

Western blot. 7.5 μg purified Type II PDE was subjected to 7.5% SDS-PAGE and subsequently transferred to nitrocellulose paper (0.45 μm). Excess protein was applied to the gels in order to visualize the lower molecular weight contaminants. Transfer was performed for 14 h (35 V, 200 mA) in a buffer consisting of 25 mM Tris-HCl (pH 8.3) 192 mM glycine/20% methanol. The nitrocellulose paper was stained with Ponceau S to verify quantitative transfer, then cut into strips for immunoblot analysis. Coomassie-stained gels showed more than 90% transfer of protein to nitrocellulose paper.

Immunoblot analysis. The immunoreactivity of the enzyme on the nitrocellulose strips was determined by incubation of the strips for 2 h in blocking buffer (100 mM Tris-HCl (pH 7.5)/0.15 M NaCl/3% BSA) and incubation with Mab5C2 (neat and 1:10 dilution) for 12 h at 4°C with gentle shaking. The strips were washed twice with blocking buffer, incubated for 2 h at room temperature with 2nd antibody (HRP-linked GAM-IgG, 1:1000), washed again with blocking buffer and then with blocking buffer without BSA (30 min). After the washing procedures, the strips were incubated for 15 min at room temperature with peroxidase color reagent (0.05% DAB) in 50 mM Tris-HCl (pH 7.5) and the color reaction developed with 0.02% hydrogen peroxide.

Results

Subcellular distribution and solubilization of Type II PDE

Differential centrifugation of rabbit brain homogenates showed that Type II PDE activity was greater than 75% particulate (Table I). This membrane-associated activity was not significantly released from the 13000 × *g* particulate fraction by hypotonic wash, EDTA-$MgCl_2$, EGTA, or high ionic strength treatment (2 M NaCl). 25–90% of the membrane PDE activity measured at 40 μM [^{3}H]cGMP substrate was solubilized by treatment with octyl-β-D-glucopyranoside, Triton X-100 and sodium deoxycholate. However, activation by cGMP was not apparent following these treatments. In contrast, 25–60% of the membrane-associated PDE activity could be released from the particulate fraction using limited trypsin treatment with retention of cGMP activation (Fig. 1) of cAMP hydrolysis.

TABLE I

SUBCELLULAR DISTRIBUTION OF RABBIT BRAIN TYPE II PHOSPHODIESTERASE

Frozen rabbit brain (6 g) was blended and homogenized in buffer A with 250 mM sucrose as described in Results. The homogenate was centrifuged at the indicated g force and the resulting supernatant and residue was assayed for Type II PDE activity at 5 μM [^{3}H]cAMP substrate in the absence and presence of 2 μM cGMP. The residues were suspended in buffer A. Total activity of the homogenate at 5 μM [^{3}H]cAMP was 1128.4 nmol/min and with 5 μM [^{3}H]cAMP+2 μM cGMP was 1863.5 nmol/min. Total activity is expressed as nmol/min. The numbers in square brackets indicate the percentage of homogenate total activity. The numbers in parentheses indicate the fold stimulation of cAMP hydrolysis by 2 μM cGMP.

Fraction	Total activity	
	5 μM cAMP	5 μM cAMP +2 μM cGMP
13000 × *g* supernatant	358.1 [31]	395.8 [21] (1.1)
13000 × *g* residue	729.0 [65]	1364.5 [73] (1.9)
100000 × *g* supernatant	397.5 [35]	392.5 [21] (1.0)
100000 × *g* residue	620.0 [55]	1323.1 [71] (2.1)

Purification of a particulate Type II (cGMP-activatable) PDE

Data from a representative preparation (400 g of rabbit brain) are represented in Table II. Similar results were obtained with ten preparations using 300–600 g of rabbit brain. The yield (2–7%) and final specific activity (25–160 U/mg) varied among preparations. All steps were performed at 4°C except where noted.

Step 1: preparation of particulate Type II PDE from rabbit brain. Frozen rabbit brains were thawed on ice and cerebelli removed. The tissue was blended in 10 volumes of buffer A (see Materials) with 250 mM sucrose using a Waring blender (three 10-s bursts, low setting) and homogenized using a Potter-Elvehjem glass homogenizer equipped with a teflon pestle (5 up and down strokes) at 1300 rpm. The homogenate was centrifuged for 60 min at 13000 × *g* in a JA-14 rotor using a Beckman J2-21 centrifuge.

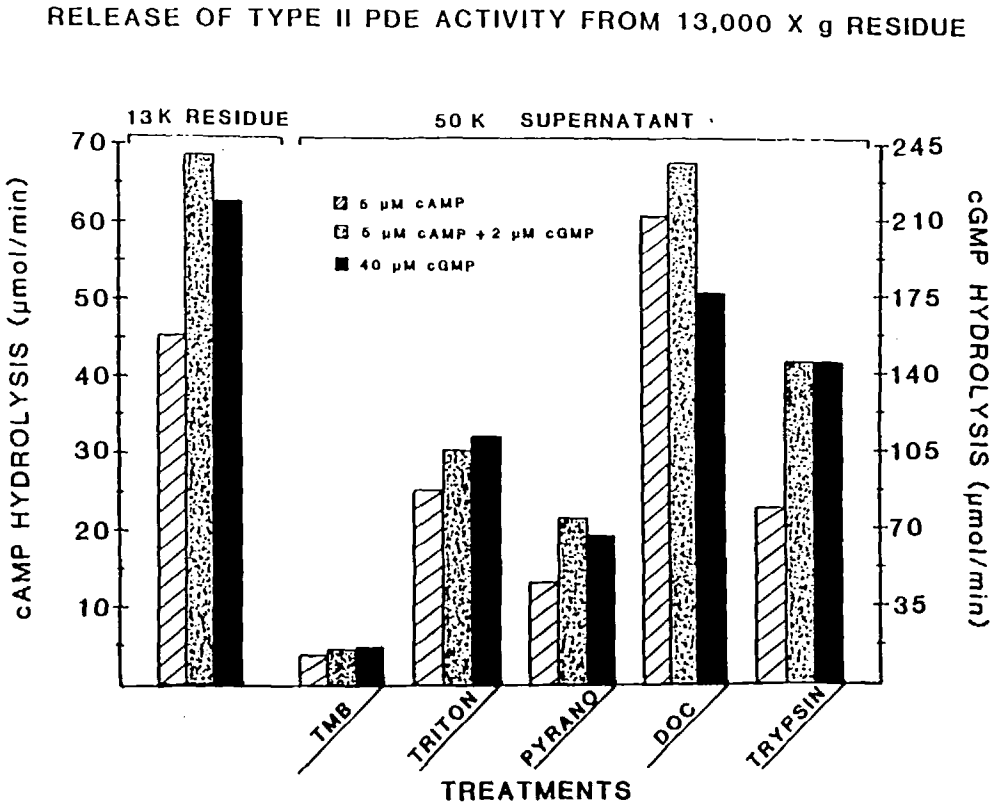

Fig. 1. Solubilization of particulate Type II phosphodiesterase. 13000×*g* particulate fraction (11.9 mg/ml) was prepared and suspended as described in the Results. The suspended residues were treated for 30 min at 4°C with the following additions; (1) 20 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/5 mM 2-mercaptoethanol (TMB) (2) 1% Triton X-100 (Triton), (3) 1% octyl-β-D-glucopyranoside (Pyrano) (4) 1% deoxycholate (DOC) and (5) 0.001% TPCK-trypsin (trypsin). After treatment, the suspension was centrifuged at 50000×*g* for 20 min and the resulting supernatants were assayed for Type II PDE activity at; 5 μM [^{3}H]cAMP, 5 μM [^{3}H]cAMP+2μM cGMP or 40 μM [^{3}H]cGMP substrate.

TABLE II

PURIFICATION OF RABBIT BRAIN PARTICULATE TYPE II PHOSPHODIESTERASE

Purification scheme using 400 g of rabbit brain. Total activity (μmol/min) and specific activity (μmol/min per mg) are with 40 μM [^{3}H]cGMP.

Fraction	Total activity (μmol/min)	Specific activity (U/mg)	Fold purification (% yield)
13 kDa residue	651	0.04	–
50 kDa supernatant	343	0.21	5 (53)
DE-52	192	0.54	14 (30)
cGMP-Sepharose	122	105	2600 (19)
Hydroxylapatite	29	134	3400 (5)

Step 2: solubilization of Type II PDE activity by limited proteolysis. The 13000 × *g* residue was suspended in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 10% (v/v) glycerol minus proteinase inhibitors using a Potter-Elvehjem glass homogenizer with a Heidolph motorized rotor (1300 rpm) driven teflon pestle (5 up and down strokes) and treated with TPCK-trypsin (1 : 500; g trypsin/g membrane protein). TPCK-trypsin was added to the suspended 13000 × *g* residue as a dry powder and the suspension stirred for 30 min. Proteolysis was terminated by the addition of an 8–10-fold excess of soybean trypsin inhibitor, TLCK (0.02 mM) and PMSF (0.43 mM). The suspension was

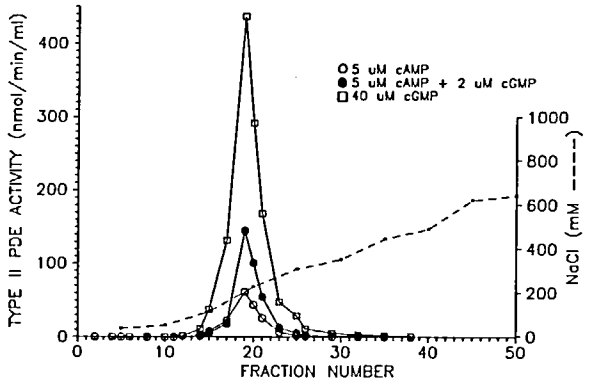

Fig. 2. DEAE-cellulose chromatography of solubilized particulate Type II phosphodiesterase. Material from step 2 (343 U/min, 1633 mg) was applied to DE-52 as described in Results. Type II PDE activity was eluted with a 0–1 M NaCl gradient at 0.75 ml/min with 3 ml fractions collected and assayed with (○) 5 μM [^{3}H]cAMP, (●) 5 μM [^{3}H]cAMP+2 μM cGMP and (□) 40 μM [^{3}H]cGMP. Peak fractions (15–22) were pooled for application to the cGMP-Sepharose affinity matrix.

centrifuged for 20 min at 50000 × *g* in a Type 45 Ti rotor using a Beckman L8-70 ultracentrifuge.

Step 3: DEAE-cellulose chromatography. The 50000 × *g* supernatant (see above) was absorbed onto DE-52 cellulose by mixing 1 ml settled resin/10 mg protein for 60 min. The supernatant and DE-52 mixture were poured into a Pharmacia column (2.6 × 40.0 cm), the column washed with 5 bed volumes of buffer A and enzyme activity was eluted with a linear 0–1 M NaCl gradient (5 bed volumes) at a flow rate of 0.75 ml/min. The peak enzyme fractions measured with either 40 μM [^{3}H]cGMP as substrate or 5 μM [^{3}H]cAMP as substrate in the absence and presence of 2 μM cGMP were pooled (Fig. 2) for application to the cGMP-Sepharose affinity matrix. Nearly equivalent specific activity Type II PDE preparations were obtained using stepwise elution from DE-52 in a glass-fritted funnel with 50, 100 and 350 mM NaCl. The 350 mM NaCl eluate contained 60–80% of the applied activity measured at 40 μM [^{3}H]cGMP substrate.

Step 4: cGMP-Sepharose affinity chromatography. The pooled DEAE fractions or 350 mM NaCl eluate was absorbed onto 10 ml of cGMP affinity matrix previously equilibrated with 20 mM Tris-HCl (pH 7.0)/2 mM dithiothreitol/1 mM EDTA/ 15 mM benzamidine (buffer B). After 60 min, the matrix was poured into a Pharmacia column (1.6 × 20.0 cm), washed with 50 bed volumes of buffer B containing 500 mM NaCl and 2 bed volumes of buffer B containing 125 mM NaCl. Type II PDE activity was eluted with 3 bed volumes of buffer B containing 125 mM NaCl and 10 mM cGMP at 23° C.

Step. 5: fast-flow hydroxylapatite chromatography. The enzyme eluted from the cGMP affinity matrix was concentrated immediately by application to a 0.50 ml (0.18 g) hydroxylapatite column equilibrated with 10 mM K_2HPO_4/KH_2PO_4 (pH 7.0)/2 mM dithiothreitol/15 mM benzamidine/ 30% ethylene glycol (buffer C). The column was washed with 5 ml of buffer C to remove cGMP and minor contaminates and Type II PDE eluted with 3–4 bed volumes of buffer C containing 100 mM K_2HPO_4/KH_2PO_4.

The overall recovery of Type II PDE activity utilizing the procedures outlined here varied from 2 to 7% with a 1200–3000-fold purification (Table II). Fig. 3 shows an SDS-polyacrylamide gel of a representative preparation. The hydroxylapatite eluate (lane 4) showed a single predominant protein band with an apparent molecular mass of 105 kDa. This band accounts for more than 90% of the protein as judged by scanning densiometric tracings of the Coomassie-stained gels (data not shown). In some preparations, lower molecular weight contaminates of 90, 56, and 40 kDa were evident. The 56 and 40 kDa proteins were removed by gel filtration with Sephadex G-100. When subjected to 7% slab gel electrophoresis under nondenaturing conditions, Type II PDE activity measured at 40 μM [^{3}H]cGMP substrate co-migrated with the major (greater than 90%) protein band observed after Coomassie staining (Fig. 4).

Properties of purified particulate Type II PDE

Initial velocity studies of the purified particulate Type II PDE showed positive homotropic cooperativity for cAMP and cGMP hydrolysis as demonstrated by curvilinear kinetic plots (Fig. 5). With cAMP as substrate, the addition of 2 μM cGMP to the enzyme assay linearized the kinetic behavior of the enzyme. This finding indicated that release of the enzyme from membranes by

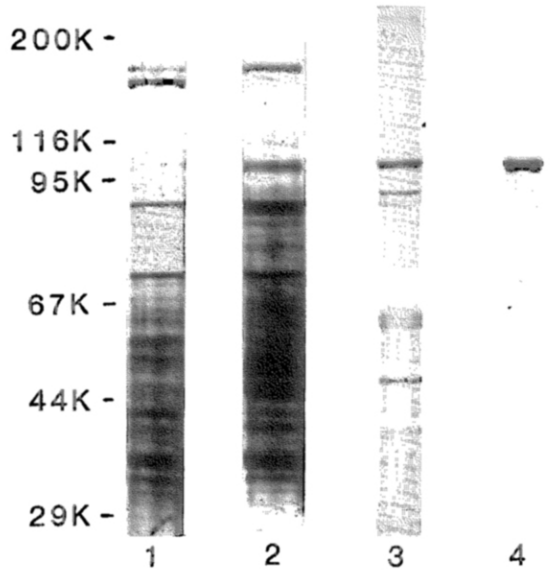

Fig. 3. SDS-polyacrylamide gel electrophoresis. Samples from each purification step were subjected to 7.5% SDS-PAGE according to Laemmli [13] and stained with Coomassie R-250 dye. Standard proteins used were; myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (95 kDa), BSA (67 kDa) and ovalbumin (44 kDa). Lane 1, 50000 $\times$ *g* supernatant (20 μg); lane 2, pooled DE-52 fractions (20 μg); lane 3, cGMP-Sepharose 10 mM cGMP eluate (10 μg); and lane 4, hydroxylapatite eluate (5 μg).

limited proteolysis preserves the kinetic properties of the enzyme equivalent to those of the cytosolic isozymes studies previously [2,3] from several bovine tissues. The substrate concentrations required for half maximum velocity were 28 μM and 16 μM for cAMP and cGMP, respectively (Fig. 6). Maximum velocities of enzyme from different preparations ranged from 25 to 160 μmol/min per mg for cAMP and cGMP. Electrophoretic profiles of enzymes with low or high maximum velocities demonstrated the same prominent protein band of 105 kDa. The apparent K_{act} for cGMP stimulation of cAMP hydrolysis at 5 μM substrate is 0.35 μM, with maximum stimulation (3–5-fold) achieved at 2 μM cGMP (Fig. 7). Greater stimulation at 2 μM cGMP was observed with decreasing cAMP concentrations (data not shown). The purified membrane-associated Type II PDE eluted from Sephacryl S-200 in the void volume, indicating a molecular size of more than 250 kDa. The purified enzyme eluted from 0.5 M Agarose and Sepharose 4B gel filtration columns as a protein with a native mass of approx. 400 kDa. The apparent molecular mass under these conditions was estimated at 380 kDa based on the Sepharose 4B gel filtration elution profile compared to five protein standards. A Stokes radius of 62.7 Å and a diffusion coefficient of 3.55 10^{-7} cm^2/s was determined by plotting the known Stokes radii and diffusion coefficients versus V_e/V_o ratios of the standards applied to the Sepharose 4B column.

Covalent labeling of type II phosphodiesterase by phosphorylation

The purified Type II PDE was found to be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase from bovine heart. Aliquots of phosphorylation reactions were subjected to 7.5% SDS-PAGE and no differences in enzyme mobility were observed by Coomassie staining. Autoradiography of the stained and dried gels revealed ^{32}P incorporation into the 105 kDa enzyme subunit (lane 1, Fig. 11). Phosphorylation reactions carried out in the absence of added

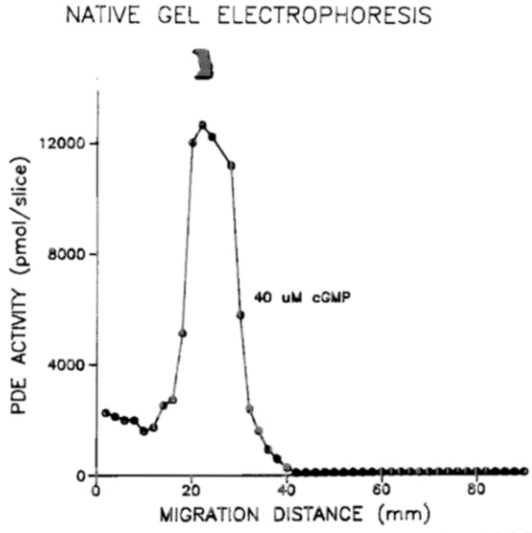

Fig. 4. Polyacrylamide slab gel electrophoresis of Type II PDE. 5 μg of purified Type II phosphodiesterase was subjected to 7% polyacrylamide gel electrophoresis under nondenaturing conditions (see Methods). One lane was stained for protein with Coomassie R-250 and another lane cut into 1-mm slices for activity analysis using 40 μM [^{3}H]cGMP as substrate (see Methods).

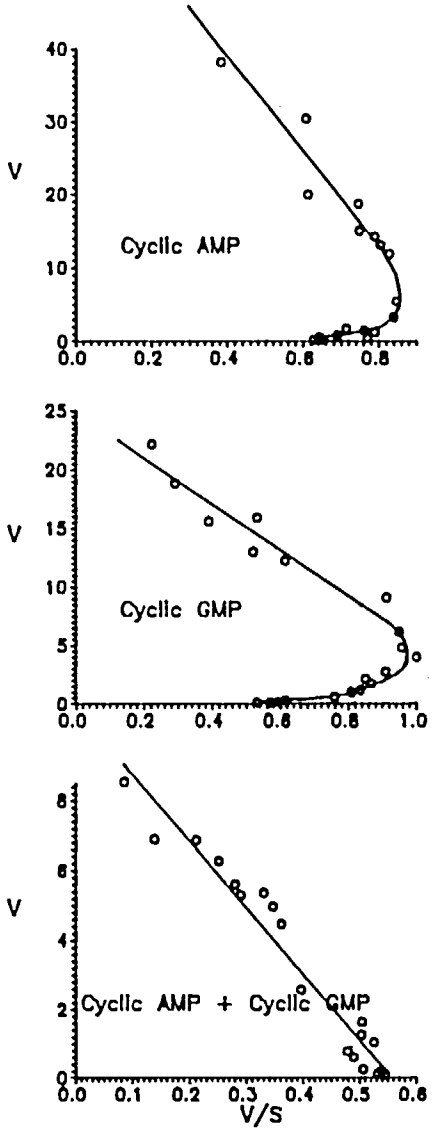

Fig. 5. Kinetic behavior of purified particulate Type II phosphodiesterase. Activity analysis was performed as described in Methods with substrate concentration of 0.2–100 μM. Eadie-Hofstee plots (v versus v/S) are shown with velocities expressed as μmol/min per mg and substrate given in μM.

catalytic subunit demonstrated trace endogenous kinase activity present in our Type II PDE preparations (data not shown).

Binding of [^{32}P]cGMP to purified particulate Type II PDE

Autoradiography following SDS-PAGE of purified particulate Type II PDE exposed to ultraviolet radiation at 254 nm for 10 min in the presence of [^{32}P]cGMP showed incorporation of ^{32}P into the 105 kDa protein band. This protein was not labeled in the presence of 1 mM cGMP. More ^{32}P was incorporated specifically into the 105 kDa enzyme subunit at pH 9.0 than at pH 6.0. Lower-molecular-mass proteins not evident by 7.5% SDS-PAGE also incorporated [^{32}P]cGMP which was displaced by 1 mM cGMP.

SUBSTRATE VERSUS VELOCITY PLOTS

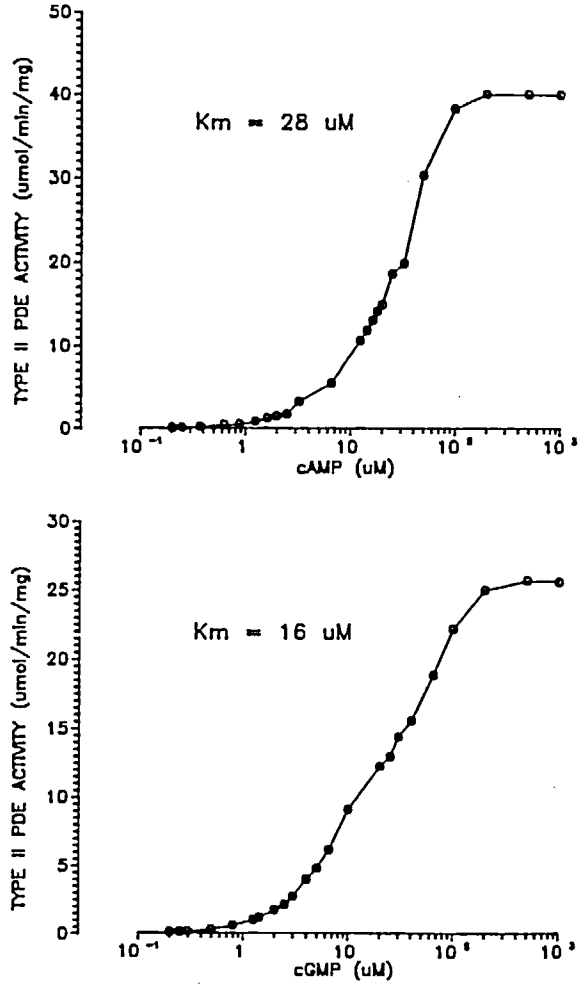

Fig. 6. Determination of $S_{0.5}$ constants for cAMP and cGMP hydrolysis by Type II PDE. Activity analysis was performed as described in Methods with substrate concentrations of 0.2–1000 μM. $S_{0.5}$ values were determined using the program Curvefit as described in Methods.

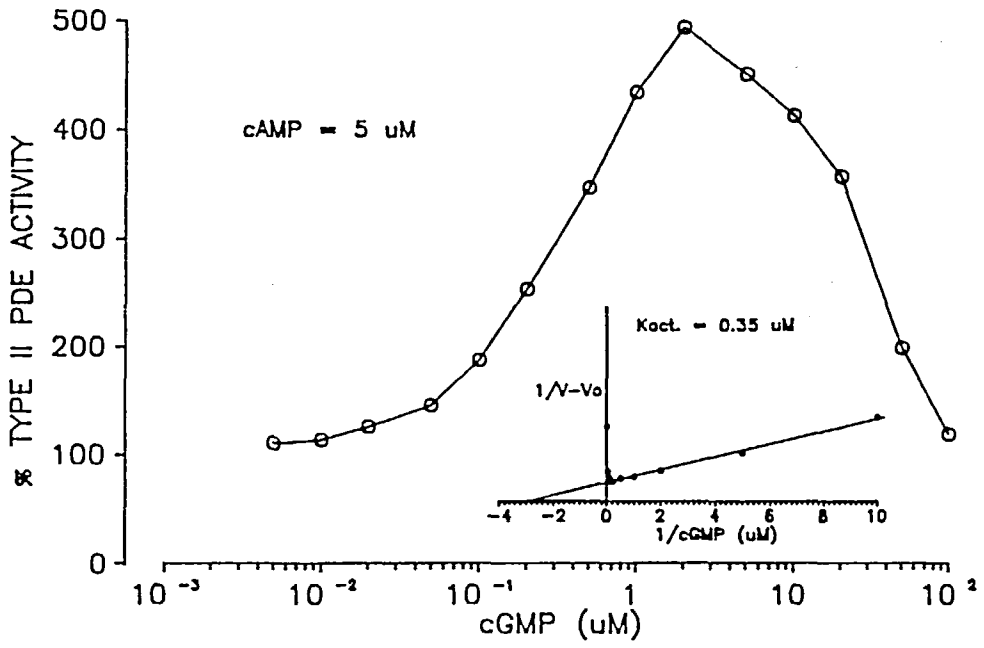

Fig. 7. Determination of the apparent K_{act} for cGMP stimulation of Type II PDE. Activity assays were performed with purified enzyme, as described in Methods, with 5 μM [^{3}H]cAMP in the presence of varying concentrations of cGMP. Activity is expressed as a percentage of Type II PDE activity at 5 μM [^{3}H]cAMP in the absence of cGMP. The apparent K_{act} was determined using a double-recipricol plot of velocity (μmol/min per mg) versus cGMP concentration with the best fit line passing through the linear portion of the plot. V, velocity at 5 μM [^{3}H]cAMP substrate in the presence of cGMP; V_o the velocity at 5 μM [^{3}H]cAMP substrate in the absence of cGMP.

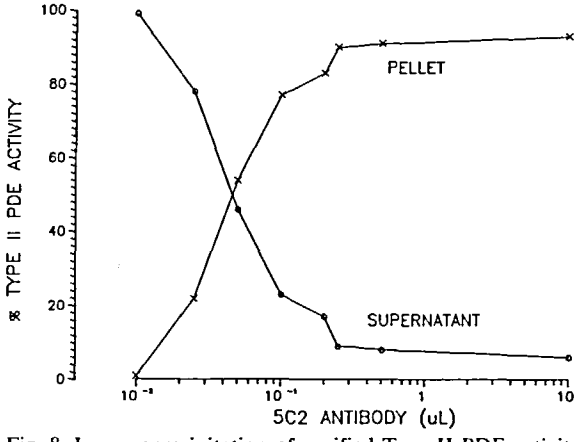

Fig. 8. Immunoprecipitation of purified Type II PDE activity by monoclonal antibody 5C2. Purified Type II PDE activity was immunoprecipitated with increasing amounts of monoclonal antibody 5C2 (29.2 mg/ml) as described in Methods. Precipitation mixtures contained 0.05 μg of Type II PDE. The resulting supernatant and pellet were assayed using 40 μM [^{3}H]cGMP substrate. Activities are expressed as a percentage of total Type II PDE activity of the precipitation mixture before centrifugation.

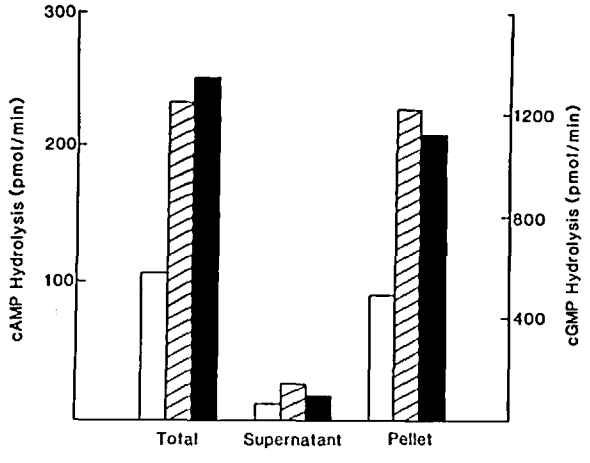

Fig. 9. Recovery of Type II PDE activity in the immune complex. 0.025 ng of purified Type II PDE was immunoprecipitated with 2.5 μg monoclonal antibody 5C2 as described in Methods. The precipitation mixture was centrifuged for 10 min at 12000$\times g$ and the resulting supernatant and pellet were assayed for Type II PDE activity using; (□) 5 μM [^{3}H]cAMP (▨) 5 μM [^{3}H]cAMP+2 μM cGMP or (■) 40 μM [^{3}H]cGMP. Total activity refers to activity measured before centrifugation.

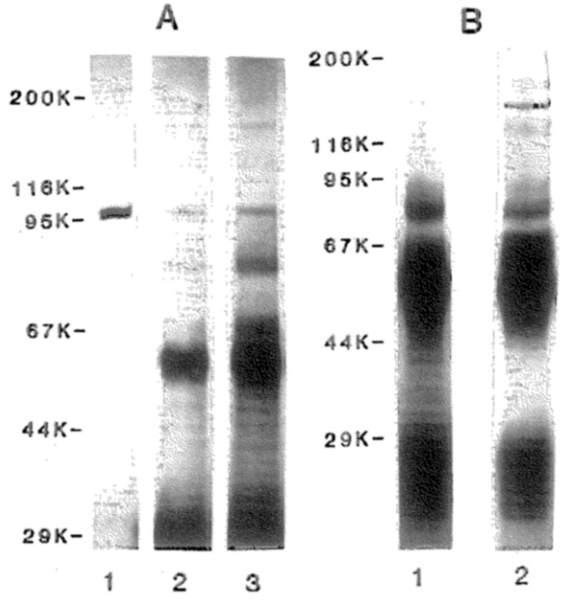

Fig. 10. Immunoprecipitation of Type II PDE protein. Protein recovered from immunopellets was analyzed by 7.5% SDS-PAGE as described in Methods. A: lane 1, 5 μg purified enzyme; lane 2, protein recovered from immunopellet using 50 μl Mab5C2-AG10 matrix; lane 3, protein recovered from immunopellet using 100 μl Mab5C2-AG10 matrix. B: antibody-matrix controls (no PDE), lane 1, 20 μg Mab5C2; lane 2, protein recovered from 100 μl of Mab5C2-AG10 matrix.

Immunoprecipitation of Type II PDE with monoclonal antibodies

Enzyme activity. In the presence of mouse IgG and second antibody, monoclonal antibody (5C2) absorbed Type II PDE activity from the solution with preservation of catalytic activity (Fig. 8). Greater than 90% of purified Type II PDE activity could be immunoprecipitated with the resulting immunopellet retaining regulation by cGMP (Fig. 9). This antibody crossreacted with the partially purified cytosolic Type II PDE from rabbit liver, but not with the partially purified Type I (calcium/calmodulin stimulated) PDE from rabbit brain (data not shown) and also immunoprecipitated solubilized Type II PDE activity in the 50000 × *g* supernatant and DE-52 eluate (data not shown).

Enzyme protein. Type II PDE protein could also be absorbed from the solution using Mab5C2-AG10 (Fig. 10), although quantitative precipitation of protein with excess matrix was not observed under conditions used to precipitate enzyme activity quantitatively. One explanation of the lack of quantitation is an undesirable coupling orientation of the Mab5C2 to the Affi-gel 10 matrix. The coupling chemistry of the Affi-gel matrix involves the nucleophilic attack of any free alkyl, aryl amine, primary amine or sulfhydryl on the carbonyl group of the *N*-hydroxysuccinimide ester derivated to the agarose bead matrix. Therefore, any of the above-mentioned groups present on the monoclonal antibody molecule can spontaneously couple with the matrix. There is reasonable probability that the antibody will couple via groups located in the antigen recognition site (F_{ab}) which would not allow for appropriate antibody–PDE interactions. An alternative explanation is that not all the 105 kDa proteins observed by 7.5% SDS-PAGE are identical, i.e., a population of 105 kDa proteins may not contain the antigenic determinant required for antibody recognition.

^{32}P-labeled enzyme. The ^{32}P-labeled Type II PDE was also recognized by the Mab5C2-AG10

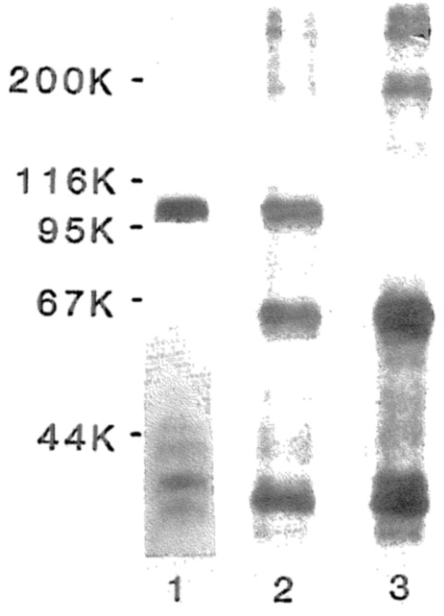

Fig. 11. Immunoprecipitation of ^{32}P-labeled protein. The ^{32}P label recovered from immunopellets (see Methods) was analyzed by autoradiography of the 7.5% SDS-gels. Lane 1, [^{32}P]PDE; lane 2, [^{32}P]PDE plus Mab5C2-AG10 matrix; lane 3, Mab5C2-AG10 matrix control (no PDE).

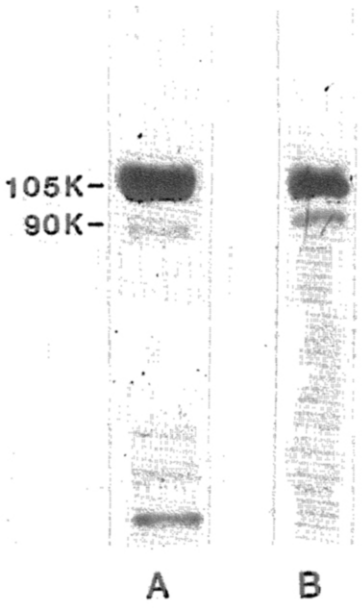

Fig. 12. Immunoblot analysis of Type II PDE. Western blot transfer with subsequent immunoblot was performed as described in Methods. Lane A, Ponceau S stained nitrocellulose strip demonstrating quantitative electrophoretic transfer; lane B, immunoblot of nitrocellulose strip showing immunoreactive protein bands at 105 and 90 kDa.

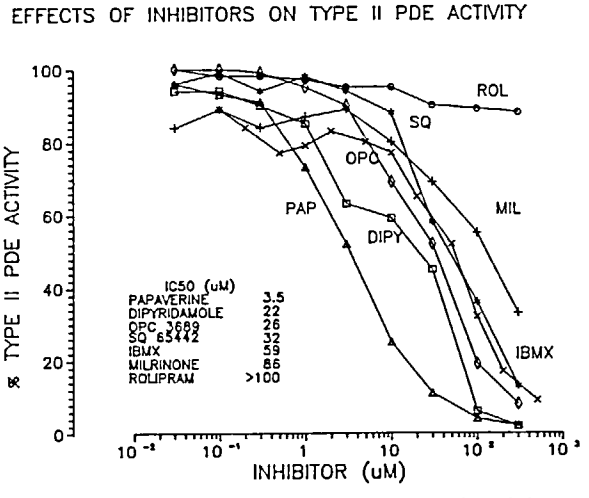

Fig. 13. Inhibition of purified Type II PDE by selected drugs. Activity analysis of purified Type II PDE was performed as described in Methods in the presence of the indicated drugs at varing concentrations. △, papaverine; □, dipyridamole; ◇, OPC-3689; *, SQ 65442; ×, IBMX; ○, rolipram; and +, milrinone. IC_{50} values were determined using the program Curvefit as described in Methods. The percent error of the IC_{50} values ranged from 5 to 25% and is that of the 95% confidence limits at IC_{50}.

(Fig. 11) as demonstrated by the recovery of ^{32}P-labeled 105 kDa protein in the immunopellet. The immunoprecipitation of labeled enzyme has not been quantitated and it is not known whether all the 105 kDa protein molecules labeled are recognized by the antibody.

Drug inhibition of Type II PDE

Several known inhibitors of PDE activity were used to characterize partially the pharmacological selectivity of the drugs for this form of the enzyme system (Fig. 13). The dose-response curves revealed a rank order of selectivity of papaverine > dipyridamole > OPC 3689 > SQ 65442 > IBMX > milrinone ≫ rolipram.

Discussion

The results of these studies show that rabbit brain Type II (cGMP-activatable) phosphodiesterase activity is predominately particulate in nature. This is in contrast to most other tissues studied thus far where this form of PDE activity is found in cytosolic fractions following homogenization and centrifugation. Greater than 75% of the homogenate Type II PDE activity of rabbit brain was tightly associated with the crude $13\,000 \times g$ pellet fraction. The enzyme appears to be an integral protein of membranes since hypotonic or high ionic strength treatments did not affect its distribution. Type II PDE activity was solubilized by several detergents; however, the released activity did not show stimulation by cGMP. It is not clear whether detergent interactions with the enzyme modify cGMP binding and/or cGMP-initiated subunit interactions or whether the detergent solubilizes latent activities of other enzyme forms that may decrease the apparent fold stimulation in the presence of cGMP. In these studies, Type II PDE activity of the $13\,000 \times g$ particulate fractions was solubilized by limited proteolytic treatment. Subsequent purification to apparent homogeneity showed that the enzyme released by proteolysis possesses physicochemical properties similar to those of soluble Type II PDE enzymes purified from bovine heart, adrenal and liver [2,3]. The rabbit brain enzyme may be membrane-associated by unique attachments similar to other membrane-associated enzymes such as acetylchol-

inestase, 5′-nucleosidase and alkaline phosphatase. These differ from classical intrinsic membrane proteins in that they are postulated to be attached by a glycoprotein arm which is anchored to the membrane by a phosphotidylinositol moiety [18–21]. Alternatively, the rabbit brain Type II PDE may have a hydrophobic region which, when cleaved by trypsin, does not affect its catalytic properties and has a minimal effect on its molecular size. Such an arrangement has been proposed by Pyne et al. [4] for the liver particulate enzyme. Additional studies are required to resolve this property of the Type II PDE in rabbit brain.

After solubilization of the enzyme, purification was essentially performed as described by Martins et al. [2], which worked satisfactorily for the solubilized enzyme. Important aspects of successful purification of Type II PDE are: (1) to minimize the time for each step of purification, (2) to wash the cGMP affinity matrix thoroughly with buffer before enzyme elution, and (3) to concentrate the enzyme immediately. These conclusions are based on our observations that the enzyme activity declined sharply if purification was not performed rapidly and/or if the enzyme was stored for any length of time in a dilute solution. Inadequate washing of the cGMP affinity matrix resulted in an increased number of contaminating proteins following elution of PDE activity with 10 mM cGMP. Since the purified Type II PDE described here is membrane derived, hydrophobic interactions with other proteins are likely and may contribute to the small complement of contaminating proteins in the cGMP-Sepharose eluate (lane 3, Fig. 3) even after extensive matrix washing. The trace proteins that co-purify with Type II PDE after the cGMP-Sepharose affinity chromatographic step, the majority of which are removed by hydroxylapatite could include: (1) other trypsin-released cGMP or Type II PDE binding proteins; (2) extrinsic membrane cGMP or Type II PDE-binding proteins; and (3) proteolytic fragments of the native Type II PDE. Enzyme activity described here was stable for at least 6 weeks when stored at $-20\,^{\circ}C$ in the hydroxylapatite elution buffer. The specific activity (approx. 160 U/mg), subunit molecular weight (105 kDa), apparent native size (approx. 380 kDa) and Stokes radius (62.7 Å) of the membrane-associated Type II PDE purified here differ markedly from the membrane-associated activity purified from rat liver membrane [4]. In addition to a lower specific activity (2–4 U/mg), the rat liver enzyme shows no positive cooperativity for cGMP hydrolysis [4], has a lower subunit molecular mass (66–67 kDa) and native mass (134 kDa). The rat liver enzyme may represent a modified form of the Type II PDE, since it differs from the cytosolic isozymes purified by others [2,3] and the particulate isozyme purified here.

The Type II PDE described in this paper has a Stokes radius of 62.7 Å and elutes from gel filtration columns (see Methods) as a protein with a apparent molecular mass of 380 kDa. This differs from the values for the cytosolic bovine calf liver enzyme as reported by Yamamoto et al. [3]. These investigators reported a native molecular mass of 201 kDa based on calculations using the sedimentation coefficient ($6.9 \cdot 10^{-13}$ s) and Stokes radius (67 Å). They suggest that the holoenzyme exists as an elongated ($f/f_0 = 1.7$) dimer of identical 102 kDa subunits. Our results show that the membrane-associated Type II PDE also consists of 105 kDa subunits, but that its native composition may be tetrameric, rather than dimeric like the cytosolic enzyme. An alternative explanation is that the brain enzyme may display anomalous elution on gel filtration due to the presence of carbohydrate moieties and an increased hydration sphere of the molecule, or because of subunit aggregation. Further experiments are required to resolve the nature of the enzyme on the membrane and its native size in solution.

Our studies show that the purified particulate Type II PDE from rabbit brain can be covalently labeled with ^{32}P using the catalytic subunit of cAMP-dependent protein kinase. Although this phosphorylation has not been fully characterized, and does not appear to be associated with any activity changes, it does reflect differences between the enzyme described here and the cytosolic isozyme purified from bovine heart. Harrison et al. [22] reported that the catalytic subunit of cAMP-dependent protein kinase did not phosphorylate Type II PDE immunoprecipitated from the cytosol of bovine heart. Their finding may be a result of the masking of the phosphorylation site by antibody binding or may reflect a fundamental

distinction between the cytosolic enzyme from bovine heart and the particulate isozyme from rabbit brain. The finding that incorporation of ^{32}P into the Type II PDE (105 kDa protein) did not correlate with 'static' activity changes, should not obscure the fact that ^{32}P was incorporated and the possibility that phosphorylation may be manifested more subtly or possibly when the enzyme is membrane-associated. Studies are being conducted with the purified and membrane-associated enzyme under phosphorylation conditions to elucidate whether phosphorylation has any biologically significant action on the Type II PDE.

Monoclonal antibodies directed against the purified particulate Type II PDE immunoprecipitated both purified and crude enzyme from rabbit brain. The antibody also crossreacted with the partially purified enzyme from rabbit liver cytosol. Enzyme protein and ^{32}P labeled enzyme protein were recovered in the immunopellet of immunoprecipitation reactions, indicating that the 105 kDa protein is associated with Type II PDE activity. The 105 kDa protein was also immunoreactive as demonstrated by immunoblot analysis. A useful property of Mab5C2 is the preservation of cGMP stimulation fo cAMP hydrolysis in the immunopellet. The antibody may be useful for Type II PDE quantification and characterization in mammalian tissues, particularly brain particulate fractions.

The 90 kDa protein which was evident to some degree in most preparations was also immunoreactive (Fig. 12). The most logical and probable explanation is that the 90 kDa protein is a proteolytic fragment of a 105 kDa subunit which contains the antigenic determinate recognized by the monoclonal antibody. Alternatively, this protein might be a lower molecular weight subunit or a membrane docking protein for the enzyme. The 90 kDa protein was not phosphorylated nor photoaffinity labeled by [^{32}P]cGMP which is of interest if it is a proteolytic fragment of the 105 kDa subunit. This would imply that a phosphorylation and cGMP-binding site may reside on a common 15 kDa peptide. Further studies utilizing peptide mapping of the 105 and 90 kDa proteins are needed to elucidate whether the 90 kDa protein is derived from the 105 kDa species.

These results show that photolysis may be used to bind [^{32}P]cGMP covalently to the purified Type II PDE. Binding is specific as evidenced by displacement of the [^{32}P]cGMP with unlabelled cGMP. We have not determined whether the catalytic or allosteric cGMP binding sites are being labeled by this procedure. From our initial studies, cGMP binding to Type II PDE appears to be greater at pH 9.0 than at pH 6.0. The use of this photoaffinity probe will enable us to identify the cGMP-binding peptide(s) located on the enzyme and compare it (them) to other cGMP binding proteins, such as cGMP-dependent protein kinase. The incorporation of [^{32}P]cGMP into the 105 kDa protein provides further evidence to support the conclusion that this protein is related to the Type II PDE.

Drug inhibitor studies using the purified particulate Type II PDE indicated a pharmacological selectivity similar to the Type II PDE purified from bovine liver cytosol as described by Yamamoto et al. [3]. Drugs selective for Type IV PDE, e.g., milrinone and rolipram, had no effect on the purified Type II enzyme.

Since cyclic nucleotides are generated by membrane-associated adenylate and guanylate cyclases, it would seem logical to impose a cellular degradative regulatory component in close proximity to their site of formation. Our finding that Type II PDE is predominantly particulate suggests the enzyme is a candidate for this regulatory component in brain tissue. Regulation of cyclic nucleotides by Type II PDE may subserve several types of control including a feedback mechanism for the fine tuning of cyclic nucleotide levels. Our studies suggest also that this feedback mechanism may involve the phosphorylation of the membrane-associated Type II PDE by a protein kinase although no activity changes were observed using the purified Type II PDE. The development of photoaffinity and monoclonal antibody probes for the analysis of Type II PDE reported here will further aid the understanding of the role of this enzyme in the regulation of cyclic nucleotides in the central nervous system.

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