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ADRENAL MEDULLARY CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

SUBCELLULAR DISTRIBUTION, PARTIAL PURIFICATION AND REGULATION OF ENZYME ACTIVITY

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Summary

The major cyclic nucleotide phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) of bovine adrenal medulla has been purified 360-fold and characterized with respect to subcellular distribution, substrate specificity and kinetic properties. When assayed at millimolar concentrations of cyclic nucleotides, only one major activity was found in this tissue, and it was localized in the 100000 × *g* supernatant. The enzyme has activity directed against both cyclic AMP (K_m = 83.8 μM; V = 126 nmol/min per mg protein) and cyclic GMP (K_m = 69.4 μM; V = 131 nmol/min per mg protein). Cyclic AMP and cyclic GMP appear to be hydrolyzed at the same catalytic site. In addition to the observations that the cyclic AMP and cyclic GMP hydrolyzing activities copurified during protein fractionation, and had identical electrophoretic behavior, each cyclic nucleotide was determined to be a competitive inhibitor of the hydrolysis of the other, with K_i values being roughly similar to their respective K_m values as substrates. The enzyme displays positive cooperative kinetic behavior when either cyclic nucleotide is the substrate; however, the cooperativity is more marked with cyclic AMP than with cyclic GMP.

Low concentrations of cyclic GMP (1 μM) activate cyclic AMP hydrolysis approximately six fold. This activation is due to a loss of cooperativity accompanied by a two fold decrease in the apparent K_m value for cyclic AMP. Cyclic IMP is a less effective activator of the enzyme. Low concentrations of *para*-chloromercuribenzoate activate cyclic AMP hydrolysis in the absence of cyclic GMP, but do not potentiate the cyclic GMP activation of the enzyme. The

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Abbreviations: EGTA, ethyleneglycol-bis-(β-amino-ethylether)-*N*,*N*′-tetraacetic acid; CDR, calcium-dependent regulator protein; *p*Cl-HgBzO$^-$, *para*-chloromercuribenzoate.

enzyme is competitively inhibited by aminophylline and noncompetitively inhibited by desipramine.

The finding that the major cyclic nucleotide phosphodiesterase of adrenal medulla is a cyclic GMP-activated cyclic nucleotide phosphodiesterase suggests that this enzyme may be important in regulating changes in the cyclic AMP: cyclic GMP ratio following cholinergic stimulation in this tissue.

Introduction

Cyclic nucleotides have been implicated as mediators of the post-synaptic actions of neurotransmitters [1,2]. Studies of the metabolism and actions of cyclic nucleotides in brain are complicated by the cellular heterogeneity of this tissue. It has been demonstrated, for example, that the adenylate cyclase of cultured neuroblastoma cells is activated by both adenosine [3] and prostaglandins [4], but not by catecholamines [4], whereas norepinephrine does activate the adenylate cyclase of glial tumor cells [5]. Adrenal medulla, which has a high degree of cellular homogeneity, offers an instructive model of the sympathetic neuron and is of potential value in studying the regulation of cyclic nucleotide metabolism and function in a well-defined neural tissue. For example, transsynaptic induction of tyrosine hydroxylase in adrenal medulla has been shown to be dependent upon changes in cyclic nucleotide levels [6]. Despite the importance of cyclic nucleotides in adrenal medulla, little information is available on the properties of the enzymes responsible for their synthesis and degradation in this tissue. We have previously reported that the major cyclic nucleotide phosphodiesterase activities in brain and adrenal medulla differ markedly with respect to regulation of enzyme activity [7], and a further study of the properties of the adrenal medullary enzyme was considered necessary to gain insight into cyclic nucleotide metabolism in this tissue. In this report the subcellular distribution, partial purification and activation of this enzyme by cyclic GMP are described.

Materials and Methods

Materials. [G-^{3}H]cyclic AMP (37.7 Ci/mmol) and [G-^{3}H]cyclic GMP (9.92 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Cyclic AMP, cyclic GMP, cyclic IMP, cyclic UMP, cyclic CMP, dibutyryl cyclic AMP, 2′:3′-cyclic AMP, 2′:3′-cyclic GMP and lyophilized 5′-nucleotidase, grade IV, from *Crotalus atrox*, were purchased from Sigma Chemical Co., St. Louis, Mo. Bio-Rad AG 1-X2 resin, 100—200 mesh, and hydroxyapatite (Bio-Gel HTP) were purchased from Bio-Rad Laboratories, Richmond, Ca. Whatman microgranular DEAE-cellulose (DE-32) was purchased from Reeve Angel, Clifton, N.J. Desipramine was generously donated by USV Pharmaceutical Corp., Tuckahoe, N.Y.

Subcellular fractionation. Bovine adrenal glands were obtained from Oscar Mayer Meat Packing Company, Madison, Wisc. and kept on ice during transport to the laboratory. Adrenal glands were slit lengthwise and medullary tissue was removed by careful dissection. All subsequent operations were performed at

4°C. Adrenal medulla (5 g) was homogenized in 8 vols. of 25 mM Tris · HCl, pH 7.4, containing 0.25 M sucrose in a Potter-Elvehjem homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 900 × *g* for 5 min to obtain a crude nuclear pellet which was resuspended in the homogenization solution and recentrifuged at 900 × *g* for 5 min. The washed nuclear pellet was resuspended in an appropriate volume of the homogenization solution. The 900 × *g* supernatants were combined and centrifuged at 12000 × *g* for 20 min to obtain a crude mitochondrial pellet. This pellet was washed by resuspension in the homogenization solution, recentrifuged at 12000 × *g* for 20 min and resuspended in the homogenization solution. The postmitochondrial supernatants were combined and centrifuged at 100000 × *g* for 60 min to obtain a microsomal pellet and a 100000 × *g* supernatant. The microsomal pellet was resuspended by homogenization in a Dounce homogenizer, using an appropriate volume of homogenization buffer.

Partial purification of adrenal medullary cyclic nucleotide phosphodiesterase

Preparation of the 100000 × g supernatant. Bovine adrenal glands were obtained from Oscar Mayer Meat Packing Company, Madison, Wisc. and kept on ice during transport to the laboratory. Medulla was scraped free from the cortex, rinsed several times with cold deionized water and either used fresh or stored frozen at −70°C until used.

Frozen adrenal medulla (300 g) was thawed quickly by briefly stirring the tissue with a small amount of deionized water at room temperature. All subsequent operations were performed at 4°C. The tissue was homogenized with two volumes of deionized water in a Waring Blendor for 1 min and then with a Brinkman Polytron (No. 7 setting) for 1 min. The homogenate was adjusted to pH 5.9 with 6 N acetic acid and centrifuged at 100000 × *g* for 60 min.

Ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant to 25% saturation while the pH of the solution was maintained at neutrality by the dropwise addition of 1 N NH_4OH. After standing for 30 min, the suspension was centrifuged at 100000 × *g* for 20 min. The pellet was discarded and the supernatant was brought to 50% saturation with respect to ammonium sulfate while the pH was maintained at neutrality. After standing for 30 min, the precipitate was collected by centrifugation at 100000 × *g* for 20 min and dissolved in a minimum volume of 20 mM Tris · HCl, pH 7.4.

Calcium phosphate gel adsorption. Calcium phosphate gel was prepared according to the procedure of Keilin and Hartree [8]. Calcium phosphate gel was slowly added to the protein solution, with stirring, to reach a ratio of 0.8 g gel to 1.0 g protein. After stirring for approximately 1 h, the gel was collected by centrifugation at 10000 × *g* for 15 min. The pellet was discarded and the supernatant was brought to saturation with respect to ammonium sulfate and allowed to stand for 30 min. The precipitate was collected by centrifugation at 100000 × *g* for 20 min and dissolved in a minimum volume of 50 mM Tris · HCl, pH 7.4. The sample was dialyzed against the same buffer with several changes.

DEAE-cellulose chromatography. DEAE-cellulose was prepared for use according to the manufacturer's directions. The resin was washed several times with 50 mM Tris · HCl, pH 7.4, containing 1 mM $MgCl_2$, 0.1 mM EGTA and 5%

glycerol and packed into a column (2.5 × 50 cm) under 8 lb/in^2 of N_2. The column was equilibrated with approximately ten column volumes of the same buffer at a flow rate of 50 ml/h. The calcium phosphate gel eluate was clarified by centrifugation at 100000 × *g* for 20 min and applied to the DEAE-cellulose column under 8 lb/in^2 of N_2. The column was washed with 500 ml of the same buffer solution before being developed with a linear NaCl gradient (900 ml) to 0.5 M NaCl in the same buffer. Fractions of 5.5 ml were collected. Assays for protein and cyclic nucleotide phosphodiesterase activity were performed on 0.05 ml aliquots of every third column fraction. The contents of tubes constituting the activity peak were pooled, concentrated to about 5 ml in a Dia-Flo apparatus (UM-10 membrane) and dialyzed against several changes of 0.01 M potassium phosphate buffer, pH 6.8. Alternatively, if preparation of the enzyme was to end at this step, the contents of tubes constituting the activity peak were pooled, dialyzed against 25 mM Tris · HCl, pH 7.4, and stored frozen at −70° C in 1 ml aliquots until used. This enzyme preparation was stable for at least 6 months when stored in this manner.

Hydroxyapatite chromatography. Hydroxyapatite (10 g Bio-Rad Bio-Gel HTP) was suspended in 100 ml 0.01 M potassium phosphate buffer, pH 6.8, and allowed to settle for 30 min. The fines were discarded and the process was repeated two more times. The final suspension was poured into a column (1 × 28 cm) and allowed to settle by gravity. The column was equilibrated with approximately 200 ml of 0.01 M potassium phosphate buffer, pH 6.8, containing 5% glycerol, at a flow rate of approximately 12 ml/h. The enzyme activity eluted from DEAE-cellulose was applied and the column was washed with an additional 40 ml of the above buffer solution. Fractions containing 1.8 ml were collected. The column was then developed with a linear gradient (330 ml) to 0.4 M potassium phosphate, pH 6.8. Aliquots (0.1 ml) were taken from every third fraction and assayed for cyclic nucleotide phosphodiesterase activity and protein content. The contents of tubes constituting the activity peak were pooled, concentrated to approximately 5 ml in a Dia-Flo apparatus (PM-10 membrane) and dialyzed against several changes of 50 mM Tris · HCl, pH 7.4. The final enzyme preparation was used immediately, rather than stored, since the enzyme activity decayed rapidly during storage at −70° C.

Cyclic nucleotide phosphodiesterase assay

Two procedures were used to measure cyclic nucleotide phosphodiesterase activity.

Method A. Cyclic nucleotide phosphodiesterase activity was measured by a modification of the method of Brooker et al. [9]. Incubations were performed in a total volume of 0.8 ml containing 25 mM Tris · HCl, pH 7.5, 2 mM $MgCl_2$, labeled cyclic nucleotide, an appropriate concentration of enzyme and any potential inhibitors or activators. The reaction was initiated by the addition of either 0.1 ml of a cyclic nucleotide solution containing 0.3 μCi of ^{3}H-labeled cyclic nucleotide or an aliquot of the enzyme solution. Reactions were performed with either saturating levels (1.2 mM), approx. K_m levels (100 μM), or low levels (1 μM), of cyclic AMP or cyclic GMP as substrate. The assay tubes were incubated with shaking at 30° C for 5—15 min and the reaction was terminated by boiling for 1 min. After cooling the tubes to room temperature, 0.1

ml of 5'-nucleotide (0.2 unit) was added and the incubation was continued at 30°C for 20 min. At the end of the second incubation, 1.0 ml of a Bio-Rad AG 1-X2 resin slurry (1 : 1 settled resin volume in deionized water), was added to bind unconverted substrate. The resin was allowed to settle and aliquots of the resulting supernatant, containing ^{3}H-labeled nucleoside, the final product of the reaction, were added to 10 ml of Triton X-100 : toluene (1 : 2) scintillation fluid [10]. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer. The resulting values were corrected for binding of nucleoside to the resin and for blanks incubated without enzyme or blanks that contained heat-inactivated enzyme.

This assay procedure was routinely used for all cyclic nucleotide phosphodiesterase determinations, except for experiments determining substrate specificity when radioactive substrates were unavailable.

Method B. This method was a modification of the procedure of Butcher and Sutherland [11]. In this assay, the first two incubation reactions were carried out exactly as for the radiometric procedure. The second incubation was terminated by the addition of 0.1 ml of cold 55% trichloroacetic acid. The contents of the tubes were then centrifuged to remove denatured protein and aliquots of the supernatant were analyzed for inorganic phosphate by the method of Chen et al. [12].

The results of the two assay procedures were identical within the limits of experimental error. All enzyme assays were performed in duplicate or triplicate, with the range between replicates being ±5%. For both of the cyclic nucleotide phosphodiesterase assay procedures, enzyme concentration and time of incubation were adjusted so that the hydrolysis of substrate was less than 25%. When determining kinetic parameters, hydrolysis of substrate was less than 10%. For all enzyme preparations both in the presence and absence of activators and inhibitors, reaction rate versus time and enzyme concentration were determined to be linear for each of the substrate concentrations used.

Preparation of Bio-Rad AG 1-X2 resin for use in the radiometric cyclic nucleotide phosphodiesterase assay

Bio-Rad AG 1-X2 resin, 100—200 mesh, (2 lb) was washed with three volumes of 1 N NaOH, followed by extensive washing with deionized water. The resin was then washed with three volumes of 1 N HCl and then repeatedly with deionized water to approx. pH 5. The resin was allowed to settle and one volume of deionized water was added to one volume of the settled resin. The resin was stored in the cold room until used.

Polyacrylamide gel electrophoresis

Disc gel electrophoresis was conducted in Tris/glycine buffer, pH 8.3, as described by Davis [13]. Acrylamide gels (7.5%) were cast in 0.5 × 6.5 cm tubes and pre-electrophoresed at 2 mA per gel for 30 min at 4°C. Protein samples were diluted with electrophoresis buffer containing 10% (w/v) sucrose and layered on the gels with a Hamilton syringe. Electrophoresis was conducted for 2 h at 2 mA per gel at 4°C with Bromophenol Blue tracking dye. Gels were frozen at −70°C for 20 min and then sliced into 1 mm sections using a gel slicer (Hoeffer Scientific Instruments). To elute enzyme activity, each slice was

incubated overnight at 4°C in the cyclic nucleotide phosphodiesterase assay buffer, minus the substrate.

Determination of kinetic constants

Values of kinetic constants were obtained by fitting the data to the overall rate equations with the aid of a computer program obtained from Dr. W.W. Cleland [14]. This program also provided an estimate of the standard error of the constants.

Protein determination

Protein was determined by the method of Lowry et al. [15] using crystalline bovine serum albumin as the protein standard.

Results

Properties of adrenal medullary phosphodiesterase

Preliminary experiments using a 1 : 8 homogenate of adrenal medulla determined that the enzyme reaction was linear with time for 20 min and with enzyme concentration in the tested range of 0.4—4 mg of protein per 0.8 ml assay volume. Enzyme activity exhibited a sharp pH optimum at pH 7.5 with large decreases in activity observed at pH values either more acidic or more basic (data not shown). Enzyme activity was increased in the presence of added divalent cation, with the optimal activity observed at 2 mM Mg^{2+} or 1 mM Mn^{2+} (Fig. 1). Concentrations of Mn^{2+} greater than 3 mM produced an inhibition of enzyme activity. The addition of 2 mM EDTA decreased enzyme activity to approximately 10% of the 2 mM Mg^{2+} level, demonstrating the necessity of the presence of a divalent cation. Activity of the enzyme with no added cation was greater than with added EDTA due to endogenous divalent cations in the homogenate.

Subcellular distribution of cyclic nucleotide phosphodiesterase activity

Adrenal medulla was fractionated by differential centrifugation and the distribution of cyclic nucleotide phosphodiesterase activity was determined (Table I). Cyclic nucleotide phosphodiesterase activity was assayed at both saturating (1.2 mM) and subsaturating (1 μM) substrate concentrations. At saturating substrate conditions greater than 90% of the total enzyme activity, using either cyclic nucleotide as substrate, was associated with the 100000 × *g* supernatant fraction. The highest enzyme specific activity was also located in this fraction. The ratio of cyclic AMP to cyclic GMP hydrolysis was approximately 1.0 for all fractions. When assayed at subsaturating concentrations of cyclic AMP, considerable activity was present in all of the fractions, although most was contained in the 100000 × *g* supernatant. These results might be indicative of enzymes present in the particulate fractions that have a lower K_m value for cyclic AMP compared to those present in the supernatant fraction. The enzyme activities associated with the particulate fractions were not further studied. When 1 μM cyclic GMP was used as the substrate, the distribution of enzyme activity was very similar to that observed at high substrate levels. Cyclic GMP was hydrolyzed faster than cyclic AMP by the 100000 × *g* supernatant fraction

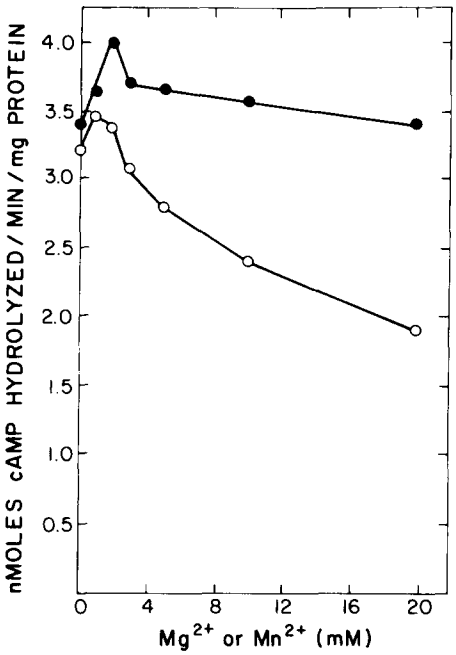

Fig. 1. The effect of divalent cations on adrenal medullary cyclic nucleotide phosphodiesterase activity. Adrenal medulla (1 g) was homogenized in 8 vols. of 25 mM Tris · HCl, pH 7.4, for 30 s in a Polytron homogenizer. Enzyme assays contained 25 mM Tris · HCl, pH 7.5, varying concentrations of either $MgCl_2$ (●———●), $MnCl_2$ (○———○), or 1.2 mg of homogenate protein and 1.2 mM cyclic AMP. Incubations were performed at 30°C for 15 min. Since 5'-nucleotidase has a requirement for Mg^{2+}, before initiating the second reaction $MgCl_2$ was added to give a final concentration of 2 mM to those tubes containing either no additional cation or 2 mM EDTA. Results reported are the mean of duplicates for a typical experiment.

at the low substrate concentration, while the reverse was true for the three particulate fractions. Sonication of the isolated fractions prior to assay did not change the activity observed. The addition of either EGTA or calcium and the calcium-dependent regulator of cyclic nucleotide phosphodiesterase also had no effect on the enzyme activities [16].

Partial purification of adrenal medullary cyclic nucleotide phosphodiesterase

A 360-fold purification of the major adrenal medullary cyclic nucleotide phosphodiesterase activity was achieved by use of conventional techniques of protein purification, including differential centrifugation, ammonium sulfate fractionation, calcium phosphate gel adsorption, DEAE-cellulose chromatography and hydroxyapatite chromatography (Table II), as detailed in Methods. The hydrolytic activities directed toward cyclic AMP and cyclic GMP copurified when assayed at saturating substrate levels, suggesting that these activities may reside in a single enzyme species. Chromatography on hydroxyapatite separated cyclic nucleotide phosphodiesterase from the greater fraction of other proteins in the sample, but the activity after purification through the hydroxyapatite step was very unstable, with greater than 50% losses of enzyme activity occurring within 24 h. Attempts to stabilize the enzyme activity by the inclusion of dithiothreitol, magnesium, glycerol, or bovine serum albumin, or

TABLE I

SUBCELLULAR DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY IN ADRENAL MEDULLA

Bovine adrenal medulla (5 g) was fractionated as described in Materials and Methods. The incubation was performed at 30°C for 15 min. Results reported are for one of three separate fractionation experiments.

Fraction	Total protein (mg)	Protein (%)	1.2 mM cAMP		1.2 mM cGMP		1 μM cAMP		1 μM cGMP	
			(nmol/min per mg)	(%)	(nmol/min per mg)	(%)	(pmol/min per mg)	(%)	(pmol/min per mg)	(%)
Homogenate	378	100	3.87	100	3.75	100	52.4	100	79.5	100
Nuclei	84.1	22.2	0.75	4.3	0.58	3.4	24.1	10.2	5.82	1.6
Mitochondria	117	31.0	0.78	6.2	0.77	6.4	23.4	13.8	7.47	2.9
Microsomes	25.3	6.7	1.68	2.9	1.72	3.1	58.9	7.5	24.1	2.0
100 000 × *g* supernatant	130	34.4	10.4	92.4	10.0	91.7	97.5	64.0	213	92.1
% Recovery		94.3		105.8		104.6		95.5		98.6

TABLE II

PURIFICATION OF ADRENAL MEDULLARY CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

A sample from each stage of purification through the DEAE-cellulose step was assayed for cyclic nucleotide phosphodiesterase activity using 1.2 mM cyclic AMP and cyclic GMP as substrates. The specific activity of the sample purified through hydroxyapatite chromatography was determined by integrating the area under the activity peak after assaying the column fractions with 1.2 mM cyclic AMP or cyclic GMP. The results reported are for one of four different purification experiments.

Fraction	Total protein (mg)	Specific activity (nmol/min per mg protein)		Total activity (nmol/min)		Fold purification	
		cAMP	cGMP	cAMP	cGMP	cAMP	cGMP
Homogenate	26 820	3.69	4.25	98 966	113 985	—	—
100 000 × *g* supernatant	13 120	6.11	7.68	80 163	100 762	1.7	1.8
25—50% $(NH_4)_2SO_4$	3 570	10.8	15.7	38 556	56 049	2.9	3.7
Calcium phosphate gel	1 340	22.0	27.9	29 480	37 386	6.0	6.6
DEAE-cellulose	49	181	230	8 869	11 270	49	54
Hydroxyapatite	3.6	1380	1530	4 968	5 508	370	360

combinations of these agents, either during or after the final chromatography step were unsuccessful.

Alternative procedures used to further purify this activity after the DEAE-cellulose chromatography step included preparative gel electrophoresis and isoelectric focusing. No activity was recovered after either of these procedures, probably because of the lability of the enzyme to further purification, and the relatively long times necessary to complete these preparative fractionation procedures.

After polyacrylamide gel electrophoresis of a sample purified through the hydroxyapatite stage, the gel was sliced and the cyclic nucleotide phosphodiesterase was eluted from the slices and assayed with 100 μM cyclic AMP as substrate. A single peak of cyclic nucleotide phosphodiesterase activity was found migrating with an R_F value of approximately 0.21. When cyclic GMP was used as the substrate, a single peak of activity was seen to be coincident with the cyclic AMP activity. Addition of calcium-dependent regulator protein to fractions obtained during electrophoresis did not alter the activity profile. Minor calcium-dependent activities which may have eluted from DEAE-cellulose with the major activity could have been detected during electrophoresis and subsequent assay in the presence of CDR and calcium. Identical activity profiles were obtained for protein samples purified through either the calcium phosphate gel adsorption or DEAE-cellulose chromatography step. The activity peak on acrylamide gels could not be unambiguously associated with a particular stained protein band.

All subsequent experiments performed used the enzyme preparation purified through the DEAE-cellulose chromatography step because of the great lability of the enzyme at higher degrees of purity. DEAE-cellulose chromatography of adrenal medullary cyclic nucleotide phosphodiesterase assayed at either 1.2 mM or 1 μM levels of cyclic AMP or cyclic GMP demonstrated the presence of one major peak of enzyme activity which eluted with 50 mM Tris · HCl, pH 7.4, containing 0.27 M NaCl.

Substrate specificity of the major adrenal medullary cyclic nucleotide phosphodiesterase

The substrate specificity of the adrenal medullary enzyme activity was determined with a preparation purified through the DEAE-cellulose chromatography step (Table III). The adrenal medullary enzyme hydrolyzed all three purine 3′:5′-cyclic nucleotides equally at saturating substrate concentrations, but showed virtually no activity towards pyrimidine 3′:5′-cyclic nucleotides. In addition, the enzyme demonstrated no detectable activity towards purine 2′:3′-cyclic nucleotides or dibutyryl cyclic AMP.

Determination of the apparent K_m values for cyclic AMP and cyclic GMP

The influence of cyclic AMP concentration on the reaction rate over a concentration range of 1.0—750 μM was measured using the enzyme purified through the DEAE-cellulose chromatography step. A double reciprocal plot of the data was non-linear and indicative of positive cooperativity. The mean apparent K_m and V values for cyclic AMP calculated from five separate determinations were 83.8 ± 5.1 μM and 126 ± 8 nmol/min per mg protein, respectively.

When cyclic GMP was used as the substrate over a range 0.75—500 μM, the double reciprocal plot was non-linear and indicative of positive cooperativity. The Lineweaver-Burk plot for cyclic GMP was consistently found to be linear over a greater concentration range than that for cyclic AMP. A Hill coefficient of 1.0 was obtained at cyclic GMP concentrations greater than approximately 5 μM and a coefficient of 1.3 obtained at lower substrate concentrations. The mean apparent K_m and V values for cyclic GMP, calculated from four separate determinations, were 69.4 ± 6.3 μM and 131 ± 14 nmol/min per mg protein, respectively.

Evidence for the hydrolysis of cyclic AMP and cyclic GMP at the same catalytic site

In addition to the observations that the cyclic AMP and cyclic GMP hydrolyzing activities coincided after electrophoretic and chromatographic procedures

TABLE III

SUBSTRATE SPECIFICITY OF THE MAJOR ADRENAL MEDULLARY CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

Incubations containing the indicated cyclic nucleotides at a final concentration of 1.2 mM and 150 μg of enzyme protein purified through the DEAE-cellulose step were performed at 30°C for 15 min. Samples were assayed by the inorganic phosphate assay of Chen et al. [12]. Values reported are the mean of two separate experiments. The mean specific activity for cyclic AMP was 163 nmol/min per mg protein.

Substrate	Activity (% of cyclic AMP activity)
Cyclic AMP	100
Cyclic GMP	105
Cyclic IMP	103
Cyclic UMP	1.5
Cyclic CMP	1.4
2′ : 3′-Cyclic AMP	0
2′ : 3′-Cyclic GMP	0
Dibutyryl cyclic AMP	0

and were purified to the same extent throughout the fractionation procedure, each cyclic nucleotide was determined to be a competitive inhibitor of the hydrolysis of the other. The apparent K_i values of the cyclic nucleotides were similar to their respective K_m values as substrates. For these experiments, substrate concentrations were chosen so as to be within the linear portion of the Lineweaver-Burk plot for the enzyme. Increasing the concentrations of unlabeled cyclic GMP inhibited the hydrolysis of tritiated cyclic AMP. A kinetic analysis of this effect (Fig. 2A) showed that cyclic GMP was a competitive inhibitor of cyclic AMP hydrolysis. The apparent K_i for cyclic GMP was deter-

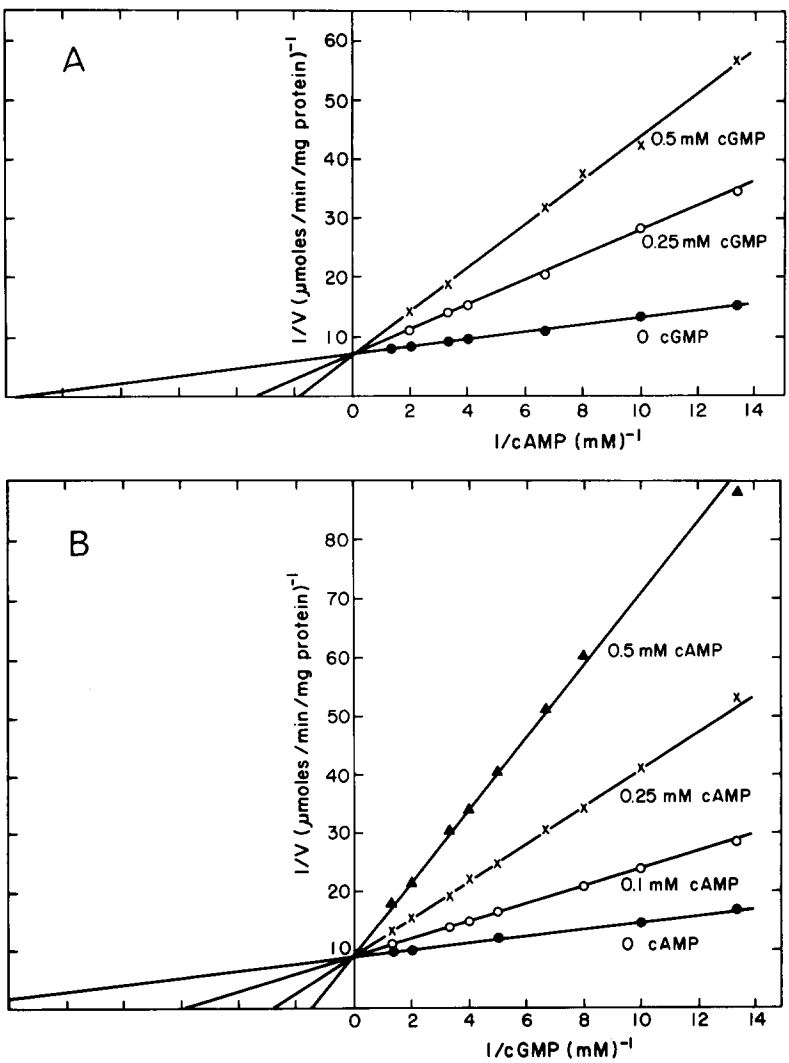

Fig. 2. A. Lineweaver-Burk plot of cyclic GMP inhibition of cyclic AMP hydrolysis. Enzyme assays contained varying concentrations of cyclic AMP, 15 μg of enzyme protein purified through the DEAE-cellulose step and either 0, (●); 0.25 mM, (○); or 0.5 mM, (X) cyclic GMP. B. Lineweaver-Burk plot of cyclic AMP inhibition of cyclic GMP hydrolysis. Enzyme assays contained varying concentrations of cyclic GMP, 15 μg of enzyme protein purified through the DEAE-cellulose step and either 0, (●); 0.1 mM, (○); 0.25 mM, (X); or 0.5 mM, (▲) cyclic AMP. The incubations were performed at 30°C for 5—15 min so that substrate hydrolysis did not exceed 10%.

mined to be 104 ± 2 μM, which was similar to the previously determined K_m for cyclic GMP (69.4 ± 6.3 μM). Cyclic AMP was also an inhibitor of cyclic GMP hydrolysis. A kinetic analysis (Fig. 2B) showed that cyclic AMP was a competitive inhibitor of cyclic GMP hydrolysis. The K_i for cyclic AMP was 51.6 ± 3.8 μM. These data suggested that the adrenal medullary preparation contained a single cyclic nucleotide phosphodiesterase which hydrolyzed both cyclic AMP and cyclic GMP at the same site.

Effect of cyclic GMP and cyclic IMP on cyclic AMP hydrolysis

The effect of increasing concentrations of two purine 3′:5′-cyclic nucleotides, cyclic GMP and cyclic IMP, on the hydrolysis of cyclic AMP was determined (Fig. 3). The experiments were performed at two substrate concentrations, 1 μM cyclic AMP and 100 μM cyclic AMP (the approximate K_m value). When the substrate concentration was low (1 μM cyclic AMP), cyclic GMP increased the hydrolysis of cyclic AMP six to seven fold. The maximum stimu-

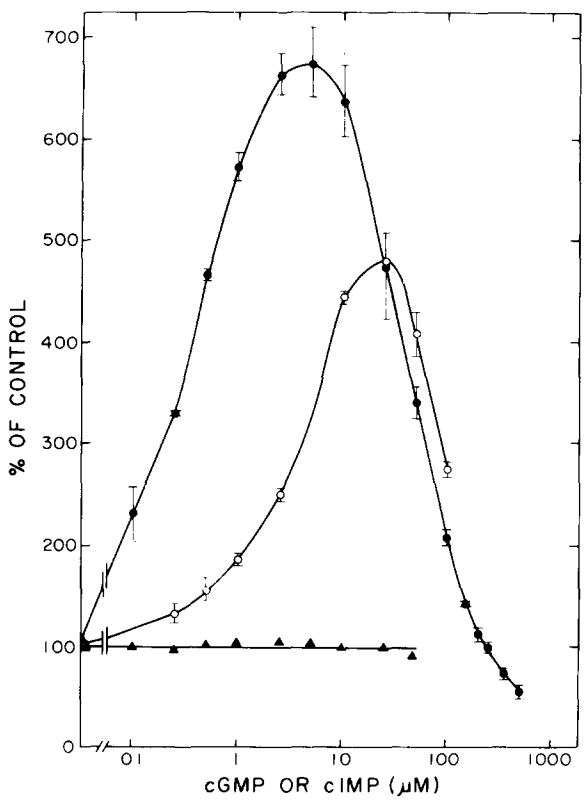

Fig. 3. Effect of cyclic GMP and cyclic IMP on cyclic AMP hydrolysis. Enzyme purified through the DEAE-cellulose step, (15 μg when 100 μM cyclic AMP was the substrate and 4 μg when 1 μM cyclic AMP was the substrate) and varying concentrations of either unlabelled cyclic GMP or cyclic IMP were incubated at 30°C for 15 min.

The results reported at 1 μM cyclic AMP concentrations are the mean ± S.E. of at least three separate determinations. The enzyme specific activity in the absence of any additions was 0.63 ± 0.02 nmol/min per mg protein. The results reported at 100 μM cyclic AMP are the mean of two separate determinations. The mean enzyme specific activity in the absence of any additions for these incubations was 72.0 nmol/min per mg protein. ●, Effect of cyclic GMP on 1 μM cyclic AMP hydrolysis; ○, effect of cyclic IMP on 1 μM cyclic AMP hydrolysis; ▲, effect of cyclic GMP on 100 μM cyclic AMP hydrolysis.

latory effect occurred in the presence of 2.5—5.0 μM cyclic GMP. The stimulatory effect of cyclic GMP was very dependent upon the concentrations of cyclic AMP used as substrate. Thus, when 100 μM cyclic AMP was used as the substrate, no stimulation of cyclic AMP hydrolysis was observed over a wide range of cyclic GMP concentrations. Concentrations of cyclic GMP greater than 100 μM were found to produce an inhibition of cyclic AMP hydrolysis as was described previously [16]. Therefore, although cyclic GMP activated cyclic AMP hydrolysis, this effect was observed only when the concentration of cyclic AMP was low. The activation by cyclic GMP was noncooperative and the concentration necessary to produce half maximal activation (K_a) when 1 μM cyclic AMP was used as the substrate was determined to be 0.41 ± 0.04 μM (Fig. 4).

Cyclic IMP was a less potent activator of cyclic AMP hydrolysis at low substrate concentrations than was cyclic GMP, with maximal stimulation (approx. 5-fold) requiring approximately 25 μM cyclic IMP. The K_a for cyclic IMP was 4.3 ± 0.7 μM. Further studies on the activation of cyclic AMP hydrolysis by other purine cyclic nucleotides were confined to the effect of cyclic GMP since this cyclic nucleotide was the more potent agent and since it has an established biological role.

Activation of cyclic AMP hydrolysis was not produced by cyclic UMP, or by possible metabolites of cyclic GMP, including 5′-GMP and guanosine [16]. To exclude the possibility that the activating effect of cyclic GMP was the consequence of raising the total substrate concentration, the hydrolysis of 1 μM cyclic GMP was examined after the addition of increasing concentrations of unlabeled cyclic AMP. Increasing concentrations of cyclic AMP inhibited cyclic GMP hydrolysis in a concentration-dependent manner [16]. These experiments suggested that the activation of cyclic AMP hydrolysis was specifically produced by cyclic GMP.

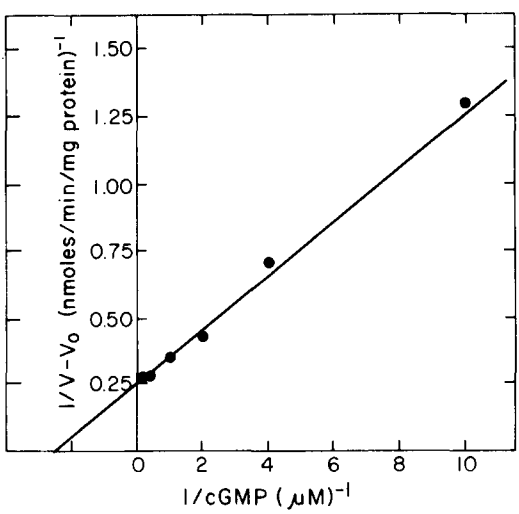

Fig. 4. Determination of the apparent activation constant (K_a) for cyclic GMP. V_0 = basal hydrolysis rate of 1 μM cyclic AMP in the absence of cyclic GMP. V = hydrolysis rate of 1 μM cyclic AMP in the presence of cyclic GMP.

Mechanism of cyclic GMP activation of cyclic AMP hydrolysis

In order to determine the mechanism of cyclic GMP activation, a Lineweaver-Burk plot was made over a wide range of cyclic AMP concentrations, both in the presence and absence of an activating concentration of cyclic GMP. As shown in Fig. 5, in the absence of cyclic GMP, the enzyme displayed positive cooperative kinetic behavior. In the presence of cyclic GMP, however, the enzyme lost its cooperativity and demonstrated Michaelis-Menton kinetics. There was no appreciable change in the V of the enzyme in the presence of cyclic GMP, but there was an approximate two fold decrease in the apparent K_m for cyclic AMP. For this experiment, the K_m for cyclic AMP in the absence and presence of cyclic GMP was 64.7 ± 7.0 μM and 34.9 ± 1.4 μM, respectively.

A Hill plot of the data in the absence of cyclic GMP was biphasic; a Hill coefficient of 1.0 was obtained at cyclic AMP concentrations above approximately 40 μM and a Hill coefficient of 1.7 was obtained at lower substrate concentrations. In the presence of cyclic GMP, however, the Hill plot was no longer biphasic and a Hill coefficient of 1.0 was obtained over the entire substrate range (Fig. 6). Thus, cyclic GMP appears to be an allosteric modifier of cyclic AMP hydrolysis.

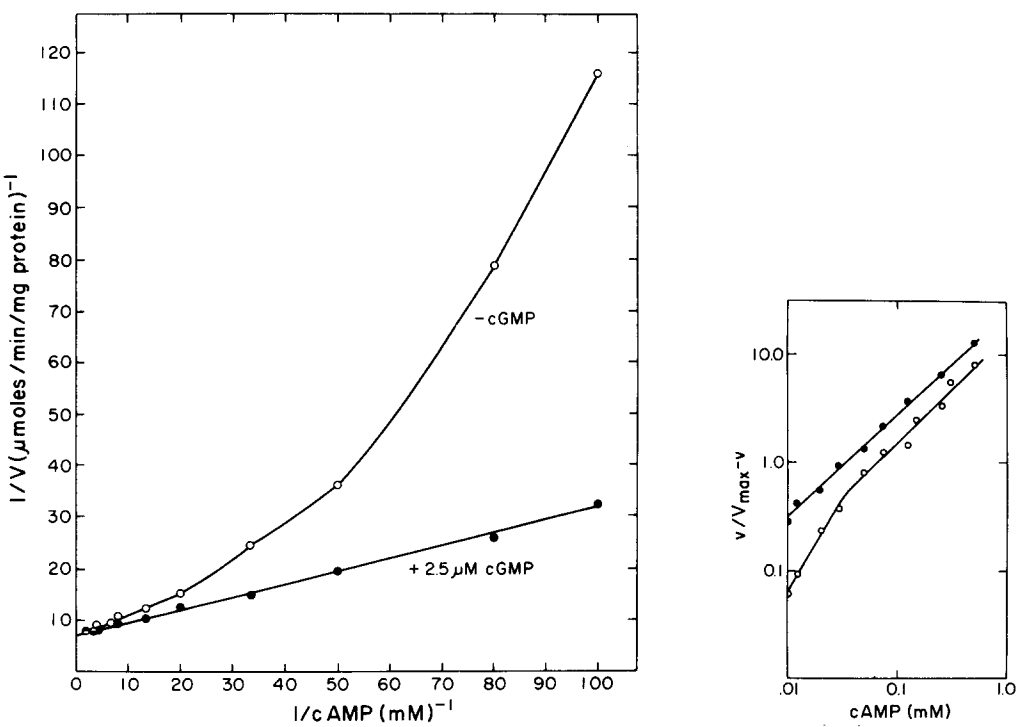

Fig. 5. Lineweaver-Burk plot of the hydrolysis of cyclic AMP in the presence and absence of cyclic GMP. Enzyme assays containing varying concentrations of cyclic AMP and 4 μg of enzyme protein purified through the DEAE-cellulose step, either in the absence (o———o) or presence (•———•) of 2.5 μM cyclic GMP were performed at 30°C for 5—15 min, so that substrate hydrolysis did not exceed 10%.

Fig. 6. Hill Plot of cyclic AMP hydrolysis in the absence (o———o) and presence (•———•) of 2.5 μM cyclic GMP.

Comparison of the cyclic nucleotide phosphodiesterase activity towards cyclic AMP and cyclic GMP under different conditions

A comparison of the catalytic rates of the adrenal medullary cyclic nucleotide phosphodiesterase preparation in the presence of saturating and low substrate concentrations and the allosteric effector is shown in Table IV. At saturating substrate conditions, cyclic AMP was hydrolyzed at approximately the same rate as cyclic GMP. This would be predicted for an enzyme having similar V values for the two substrates. At 1 μM substrate concentrations, however, cyclic AMP was hydrolyzed at only one-third of the rate of cyclic GMP. When cyclic AMP hydrolysis was measured at 1 μM substrate concentrations, in the presence of activating concentrations of cyclic GMP, cyclic AMP was hydrolyzed at almost twice the rate of cyclic GMP.

Effect of pCl-HgBzO$^-$ on cyclic nucleotide phosphodiesterase activity

As shown in Table V, incubation of the enzyme preparation with low concentrations of the sulfhydryl blocking reagent, *p*Cl-HgBzO$^-$, increased basal cyclic AMP hydrolysis to almost the same extent as the allosteric effector, cyclic GMP. The addition of *p*Cl-HgBzO$^-$, however, did not alter the activity of the enzyme in the presence of activating concentrations of cyclic GMP. Thus, when the enzyme was maximally activated by cyclic GMP, it could not be further activated by modification of sulfhydryl groups. At higher concentrations of *p*Cl-HgBzO$^-$, the basal activity, as well as the cyclic GMP-stimulated activity, decreased.

Effect of aminophylline and desipramine on cyclic nucleotide phosphodiesterase activity

Aminophylline inhibited the adrenal medullary cyclic nucleotide phosphodiesterase activity, with approximately 700 μM aminophylline producing 50% inhibition of enzyme activity. A double reciprocal plot of the effect of aminophylline on enzyme activity over a range of substrate concentrations, chosen so as to be within the linear portion of the Lineweaver-Burk plot for this enzyme, indicates that aminophylline is a competitive inhibitor of the enzyme activity, suggesting that aminophylline is interacting at the catalytic site. The K_i for aminophylline was 352 ± 29 μM.

TABLE IV

COMPARISON OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY TOWARDS CYCLIC AMP AND CYCLIC GMP UNDER DIFFERENT CONDITIONS

Enzyme assays contained the indicated concentrations of labeled substrates, in either the presence or absence of 5 μM unlabeled cyclic GMP, and an appropriate concn. of enzyme purified through the DEAE-cellulose step. Incubations were performed at 30°C for 15 min.

Substrate concentration	Cyclic nucleotide phosphodiesterase activity (nmol/min per mg protein)		
	cAMP	cGMP	cAMP +5 μM cGMP
1.2 mM (saturating)	178	167	—
1 μM	0.75	2.48	4.59

TABLE V

EFFECT OF *p*Cl-HgBzO$^-$ ON CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY

Enzyme assays contained 1 μM labeled cyclic AMP, varying concentrations of *p*Cl-HgBzO$^-$ and 5 μM cyclic GMP where indicated. The reaction was initiated by the addition of 4 μg of enzyme protein purified through the DEAE-cellulose step and continued for 15 min at 30°C. The results are the mean of triplicates.

*p*Cl-HgBzO$^-$ (μM)	Cyclic nucleotide phosphodiesterase activity				Activity ratio $\left(\frac{+\text{cGMP}}{-\text{cGMP}}\right)$
	—Cyclic GMP		+Cyclic GMP		
	(nmol/min per mg)	(% control)	(nmol/min per mg)	(% control)	
0	0.62	100	3.48	100	6.42
5	2.19	353	3.74	94.0	1.71
10	2.72	439	3.77	94.7	1.39
25	3.00	484	3.50	87.9	1.17
50	3.12	503	3.75	94.2	1.20
100	3.31	534	3.37	84.7	1.02
250	3.07	495	2.94	73.9	0.96
500	2.71	437	2.21	55.5	0.82

Desipramine inhibited enzyme activity with approximately 900 μM desipramine producing 50% inhibition of enzyme activity. Kinetic analysis of the inhibition revealed it to be noncompetitive. Re-plots of both the slope and intercept were parabolic indicating that inhibition was caused by a combination of at least two inhibitor molecules. The parabolic nature of the re-plots and use of only three inhibitor concentrations prevented calculation of the K_{is} and K_{ii}.

Discussion

When assayed at millimolar concentrations of cyclic AMP and cyclic GMP, adrenal medullary cyclic nucleotide phosphodiesterase activity was found to be almost totally located in the 100000 × *g* supernatant. At micromolar levels of substrate, the activity directed against cyclic GMP was also almost entirely soluble, whereas significant cyclic AMP hydrolysis was also found in the nuclear, mitochondrial and microsomal fractions. These data suggest that there is a low K_m cyclic AMP phosphodiesterase(s) which is membrane-bound. Cyclic nucleotide phosphodiesterases with these characteristics have been described for other tissues [17].

Fractionation of the adrenal medullary supernatant enzyme activity by DEAE-cellulose chromatography revealed one major activity peak. A partially purified enzyme preparation had similar K_m values for cyclic AMP and cyclic GMP and hydrolyzed the two cyclic nucleotides at equal rates at saturating substrate conditions. Cyclic AMP and cyclic GMP are probably hydrolyzed at the same catalytic site since each cyclic nucleotide is a competitive inhibitor of the hydrolysis of the other and since their K_i values were similar to their respective K_m values as substrates. The enzyme displayed positive cooperative kinetic behavior for both substrates, although this was only demonstrable at much lower substrate concentrations for cyclic GMP than for cyclic AMP. The great

lability of the enzyme past the DEAE-cellulose chromatography step prevented further purification.

In contrast to the complicated isoenzyme patterns seen upon chromatography of the supernatants from other tissues [17,18], this adrenal medullary enzyme is responsible for almost all of the 100000 × *g* supernatant activity when assayed at saturating substrate. Therefore, adrenal medulla serves as an ideal tissue for the isolation of and studies of the regulation of this isoenzyme.

The adrenal medulla is often employed as a model sympathetic nervous tissue, and the results presented here demonstrate differences between the cyclic nucleotide phosphodiesterase activity of adrenal medulla and brain. The subcellular distribution data of the adrenal medulla contrast with the distribution of brain cyclic nucleotide phosphodiesterase activity, which when assayed at millimolar levels of substrate was only 35% soluble [16]. We have previously reported that the major cyclic nucleotide phosphodiesterase of brain and adrenal medulla differ with respect to elution behavior on DEAE-cellulose and their calcium dependence [7]. The major adrenal medullary enzyme is calcium-independent, whereas maximum brain enzyme activity depends upon the simultaneous presence of calcium and a calcium-dependent regulatory protein [7]. In addition, the two enzymes differ with respect to their substrate affinities and kinetic behavior. The brain enzyme has a lower K_m for cyclic GMP than cyclic AMP [16,19], whereas the adrenal medullary enzyme has equal K_m values for the two cyclic nucleotides. The adrenal medullary enzyme also displays positive cooperative kinetic behavior, whereas the brain enzyme does not [16,19].

As judged by both Hill and double reciprocal plots, adrenal medullary cyclic nucleotide phosphodiesterase displayed positive cooperative kinetic behavior for both cyclic AMP and cyclic GMP, although this was only demonstrable at substrate concentrations much lower for cyclic GMP than for cyclic AMP. Low concentrations of cyclic GMP and, to a lesser extent, cyclic IMP, activated the hydrolysis of cyclic AMP at low (1 μM) cyclic AMP concentrations. The activation of cyclic AMP hydrolysis by cyclic GMP was due to a loss of cooperatively accompanied by a two fold decrease in the K_m of the enzyme for cyclic AMP. The activation of cyclic AMP phosphodiesterase activity by low concentrations of cyclic GMP, while not previously reported in adrenal medulla, has been seen in other tissues. Beavo et al. [20], Russell et al. [21] and others have reported similar effects of cyclic GMP on hepatic cyclic AMP phosphodiesterase; this effect was seen in heart by Terasaki and Appleman [22] and in thymic lymphocytes by Franks and MacManus [23]. Thus, the presence of a cyclic GMP stimulated cyclic AMP phosphodiesterase appears to be a common feature of many tissues.

The simplest model consistent with these observations suggesting that cyclic AMP and cyclic GMP are hydrolyzed at the same catalytic site, would be an enzyme having both a catalytic site and a regulatory site. The catalytic site would have similar affinities and hydrolytic rates for cyclic AMP and cyclic GMP. Although the regulatory site could also bind both cyclic nucleotides, it would have a greater affinity for cyclic GMP. The greater affinity of the regulatory site for cyclic GMP would be predicted from the fact that the Lineweaver-Burk plot for cyclic GMP was linear over a much larger concentration range than

for cyclic AMP and from the fact that cyclic GMP activated the hydrolysis of cyclic AMP at low cyclic AMP concentrations. According to this model, either cyclic nucleotide could serve as a regulator of its own hydrolysis, since increase in the concentration of a given cyclic nucleotide would increase the proportion of regulatory sites occupied. In addition, when cyclic AMP concentrations were decreased to a level where the majority of regulatory sites were unoccupied, cyclic GMP would activate cyclic AMP hydrolysis. The activating effect of the sulfhydryl blocking reagent, *p*Cl-HgBzO$^-$, might have been produced by a functional dissociation of the regulatory site. Such an effect has been demonstrated with other allosteric enzymes [24]. Confirmation of this model, however, requires further enzyme purification.

The effects of known cyclic nucleotide phosphodiesterase inhibitors were also examined. The major adrenal medullary enzyme was competitively inhibited by aminophylline. Desipramine, a tricyclic antidepressant, has been shown to be a noncompetitive inhibitor of the calcium-sensitive cyclic nucleotide phosphodiesterase of porcine brain by specifically antagonizing the activation of this enzyme by calcium and the calcium-dependent regulator [16,25]. Based on the mode of action, and the observation that the calcium-sensitive cyclic nucleotide phosphodiesterase of porcine brain was not activated by cyclic GMP when assayed either in the presence or absence of calcium and the calcium-dependent regulator (CDR) [16], desipramine was not anticipated to be an inhibitor of the adrenal medullary enzyme. Desipramine, however, was found to be a noncompetitive inhibitor of the adrenal medullary activity. The fact that desipramine inhibited both a CDR-dependent and a CDR-independent cyclic nucleotide phosphodiesterase indicates that the particular isoenzyme present in a given tissue cannot be predicted from inhibitor studies alone.

The physiological significance of cyclic GMP regulation of adrenal medullary cyclic nucleotide phosphodiesterase is not known. Although the intracellular levels of cyclic nucleotides are not precisely known, the cyclic AMP concentration has been estimated to be approximately 1 μM [17]. In the bovine adrenal medulla, the ratio of cyclic AMP to cyclic GMP is approximately nine [26]. Since the K_m of the adrenal medullary cyclic nucleotide phosphodiesterase is 70—80 μM the enzyme is probably not saturated with respect to substrate in vivo. In addition, because of its cooperative kinetic properties the enzyme has a lower hydrolytic rate at low substrate levels. Data in the present study indicate that at the estimated physiological levels of cyclic nucleotides, cyclic GMP would accelerate the enzymatic hydrolysis of cyclic AMP. A concentration of cyclic GMP as low as 0.1 μM could produce a two-fold stimulation of cyclic AMP hydrolysis at a 1 μM substrate concentration.

The "Yin-Yang" or dualism hypothesis of Goldberg [27] suggests that cyclic AMP and cyclic GMP may have antagonistic roles in biological regulation. If cyclic AMP and cyclic GMP do act in opposition to one another, a change in the relative proportion of one cyclic nucleotide to the other may be more important than the absolute change in the concentration of only one of the cyclic nucleotides in mediating these antagonistic actions.

The regulatory properties of this enzyme make it a potential candidate for regulating the ratio of cyclic AMP to cyclic GMP within the chromaffin cell. Cholinergic stimulation of this tissue has been reported to cause increases in the

intracellular levels of cyclic GMP due to stimulation of muscarinic receptors [6]. Intracellular increases in cyclic GMP might serve to stimulate cyclic AMP hydrolysis by this cyclic nucleotide phosphodiesterase and in doing so, further increase the ratio of cyclic GMP to cyclic AMP. Guidotti and Costa [28] have reported that carbamylcholine does, in fact, cause an increase in the ratio of cyclic GMP to cyclic AMP in adrenal medullary slices, which is consistent with this hypothesis.

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