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STUDIES ON CYCLIC NUCLEOTIDES IN THE ADRENAL GLAND

III. PROPERTIES OF CYCLIC AMP- AND GMP-DEPENDENT PROTEIN KINASES IN THE ADRENAL GLAND

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SUMMARY

Studies have been made on the cyclic nucleotide-binding proteins and cyclic nucleotide-dependent protein kinases in the cortex and the medulla of bovine adrenal glands. The protein kinase from the cortex was more responsive to adenosine 3′,5′-monophosphate (cyclic AMP) than to guanosine 3′,5′-monophosphate (cyclic GMP), with an apparent K_m of 0.02 μM for cyclic AMP and of 0.73 μM for cyclic GMP. The enzyme from the medulla, however, was activated to a similar extent by cyclic AMP anp GMP, with an apparent K_m of 0.17 μM for cyclic AMP and of 0.18 μM for cyclic GMP. Since the enzyme from the medulla was responsive to cyclic GMP as well as to cyclic AMP, the biological significance of cyclic GMP on the medullary enzyme was discussed.

That the activity of cyclic AMP-dependent protein kinases from the cortex is higher than that of the medullary enzyme is due to differences in the cyclic AMP-binding activity of the regulatory proteins associated with the enzyme.

INTRODUCTION

In previous experiments [1, 2] on the rat adrenal, where the gland was divided into the zona fasciculata-reticularis (the decapsulated gland), the glomerulosa (the capsular gland) and the medulla, adrenocorticotropin or immobilization stress produced an equally great increase in cyclic AMP levels in the two cortex zones (the decapsulated and capsular gland), but no changes in the medulla. Differences of the adenyl cyclase activity between the cortex and the medulla promoted further studies on the properties of the receptor protein bound to cyclic nucleotide-dependent protein kinase in these zones. The present paper presents studies on the mechanism of action of cyclic AMP and GMP on cyclic nucleotide-binding proteins and protein kinases from the cortex and the medulla of the bovine adrenal gland.

MATERIAL AND METHODS

Bovine adrenal glands, obtained fresh from Teikokuzoki, Co. Ltd., Tokyo, Japan were divided into three parts; the capsular zone with glomerulosa, the decapsulated zone with fasciculata-reticularis and the medullary zone. Since the previous experiments [3, 4] on the kinetics analysis of the protein kinase reported no differences in properties of cyclic AMP-binding proteins and cyclic AMP-dependent protein kinases between the decapsulated and the capsular gland, the tissue from the decapsulated gland was mainly referred to as the cortex in this paper.

Cyclic AMP was generously donated by Seisin Seiyaku, Co., Ltd., Tokyo, Japan. Cyclic[^{3}H]AMP with a specific activity of 20.7 Ci/mmole and [γ-^{32}P]ATP with specific activities of 1.0–2.8 Ci/mmole, were purchased from the Radiochemical Centre, Amersham, England. Cyclic[^{3}H]GMP (3.5 Ci/mmole) was from New England Nuclear, Boston, Mass. Histone from calf thymus (Type II) was from Sigma Chemical Co., St. Louis., Mo. Protein kinases and cyclic nucleotide-binding proteins from three zones were partially purified by a modification of the method of Kumon et al. [5].

15 g tissue were homogenized with 5 vol. of 0.25 M sucrose, 50 mM KCl, and 1 mM $MgCl_2$ in 50 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at 15 000 $\times$ g for 15 min followed by a 100 000 $\times$ g centrifugation of the supernatant for 120 min. An $(NH_4)_2SO_4$ precipitation and adsorption to calcium phosphate gel was performed as previously described [3, 4]. The eluate of 0.2 M potassium phosphate buffer from calcium phosphate gel was again dialyzed against a 10 mM Tris–HCl buffer, pH 7.5, containing 10% glycerol and 6 mM 2-mercaptoethanol. The enzyme solution was equilibrated with 5 mM NaCl in Tris–HCl buffer, pH 7.5, containing glycerol and 2-mercaptoethanol. Elution was carried out by application of 100 ml of 5 mM NaCl, followed by a 300 ml of linear concentration gradient from 50–500 mM NaCl in Tris–HCl buffer, pH 7.5, containing glycerol and 2-mercaptoethanol. An assay of labeled cyclic nucleotides binding to the protein was made by the method of Walton and Garren [6]. The reaction mixture (0.30 ml) contained 50 mM potassium phosphate buffer, pH 6.5, 8 mM theophylline, $5 \cdot 10^{-7}$ M cyclic [^{3}H]AMP or $3 \cdot 10^{-6}$ M cyclic[^{3}H]GMP, and a protein preparation. Incubation was for 1 h at 0 °C. The reaction was terminated by dilution with 1.0 ml of ice-cold 25 mM Tris–HCl buffer, pH 8.0, containing 10 mM $MgCl_2$.

A millipore filter (HA 0.45 μm, 25 mm) was used to separate free and protein-bound ^{3}H-labeled cyclic nucleotides. The protein kinase assay was based on the method of Miyamoto et al. [7]. The incubation medium contained 10 μmoles of sodium glycerol phosphate buffer, pH 6.0, 0.6 mg of histone, 0.9–1.2 nmoles of [γ-^{32}P]ATP, 2 μmoles of magnesium acetate, 0.4 μmole of theophylline, 0.06 μmole of ethylene glycol (β-aminoethyl ether)-N,N'-tetraacetic acid, with or without $5 \cdot 10^{-6}$ M cyclic AMP or cyclic GMP. After incubation at 30 °C for 15 min, the reaction was stopped by the addition of 4 ml of ice-cold 7.5% trichloroacetic acid, followed by 0.2 ml of 0.63% bovine serum albumin. The precipitate was washed three times with 5% trichloroacetic acid and assayed for radioactivity with a liquid scintillation spectrometer (Aloka, Tokyo, Japan).

Protein determination was by the method of Lowry et al. [8]. One unit of the enzyme activity was expressed as the amount of the enzyme incorporating 1 pmole of ^{32}P from [γ-^{32}P]ATP into the recovered protein in 15 min.

RESULTS

DEAE-cellulose chromatograms of protein kinase and cyclic nucleotide-binding proteins from the cortex and the medulla are shown in Figs 1 and 2. Two peaks of the cyclic AMP-dependent protein kinase, associated with cyclic AMP-binding protein were observed. Figs 1 and 2 also show the effect of cyclic GMP on cyclic AMP-dependent protein kinases and the binding of cyclic[^{3}H]AMP to the binding protein associated with cyclic AMP-dependent protein kinases.

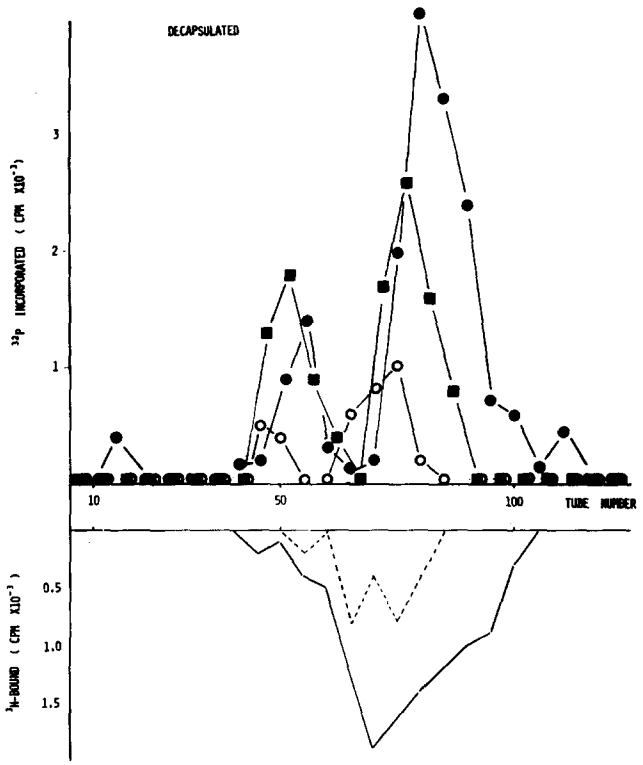

Fig. 1. DEAE-cellulose column chromatograms of protein kinases and cyclic nucleotide-binding proteins from the cortex (the decapsulated gland). Assay conditions of protein kinase activities in the presence of cyclic AMP (●—●), cyclic GMP (■—■), or in the absence of $5 \cdot 10^{-6}$ M cyclic nucleotides (○—○), and cyclic AMP (———) and GMP-binding protein (– – –) were as described in the text.

Cyclic GMP appeared to bind with the cyclic AMP-binding protein and also to stimulate the cyclic AMP-dependent protein kinase. Effects of varying concentrations of cyclic AMP and GMP on protein kinase activities of the cortex and the medulla are shown in Figs 3 and 4. The cortical enzyme had a lower K_m value (0.02 μM) for cyclic AMP than did the medulla enzyme (0.17 μM) confirming earlier results [4]. The comparison of the protein kinase activity between the cortex and the medulla at various cyclic AMP concentrations is shown in Fig. 5. Enzyme activity

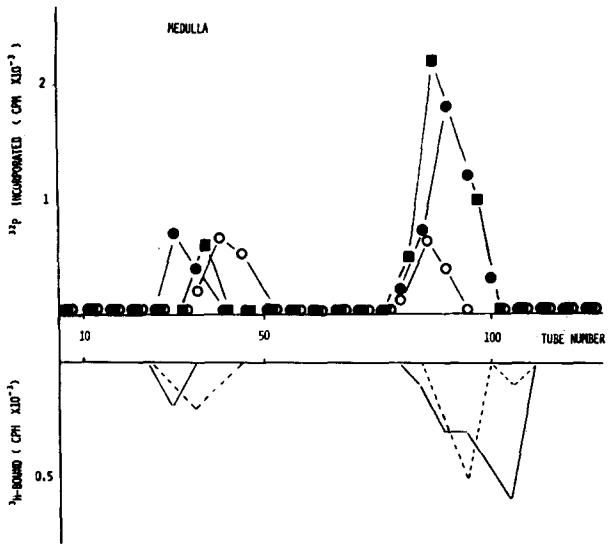

Fig. 2. DEAE-cellulose column chromatograms of protein kinases and cyclic nucleotide-binding proteins from the medulla. Assay conditions of protein kinase activities in the presence of cyclic AMP (●—●), cyclic GMP (■—■) or absence of $5 \cdot 10^{-6}$ M cyclic nucleotides (○—○), and cyclic AMP (———) and GMP-binding protein (– – –) were as described in the text.

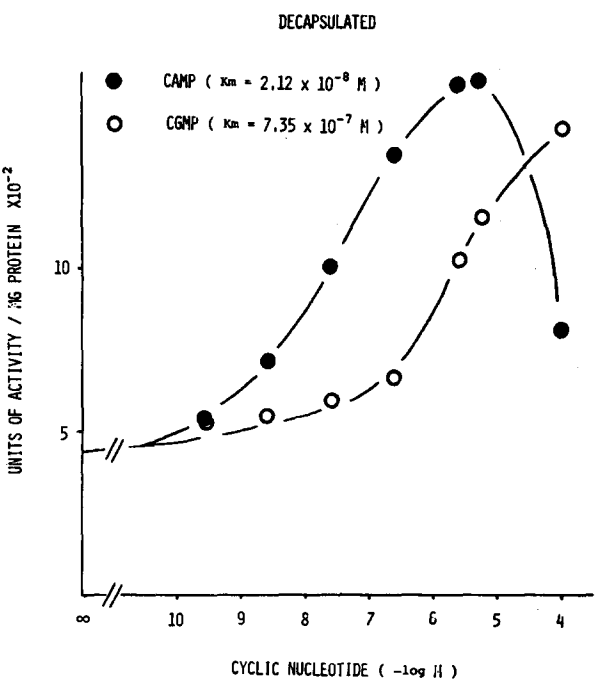

Fig. 3. Effect of varying concentrations of cyclic AMP (●—●) and GMP (○—○) on protein kinase activities of the cortex (the decapsulated gland). Assay conditions were as described in the text, except for the variation in cyclic nucleotides concentration.

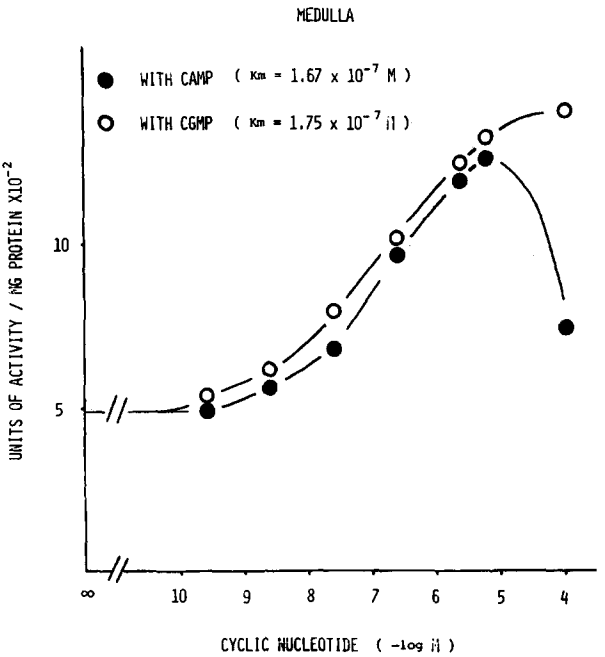

Fig. 4. Effect of varying concentrations of cyclic AMP (●—●) and GMP (○—○) on protein kinase activities of the medulla. Assay conditions were as described in the text.

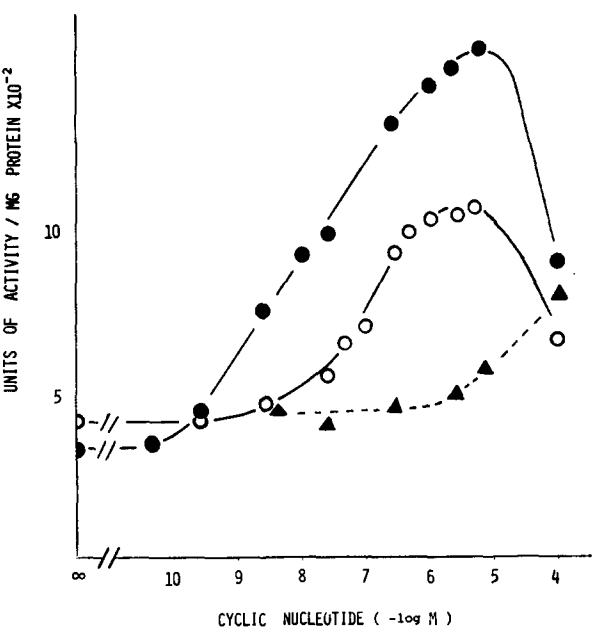

Fig. 5. Comparison of protein kinase activities responding to various concentrations of cyclic AMP and dibutyryl cyclic AMP (▲---▲) between the cortex (the decapsulated glnd, ●—●) and the medulla (○—○). Assay conditions were as described in the text.

was higher in the cortex. It was of interest that the K_m value (0.18 μM) for cyclic GMP of the medulla enzyme was lower than that of the cortex enzyme (0.73 μM). The K_m value of the cortex enzyme for cyclic GMP is 35 times greater for cyclic AMP (Fig. 3). The K_m values of the medullary enzyme, however, were the same for cyclic AMP (0.17 μM) and cyclic GMP (0.18 μM) (Fig. 4). Cyclic AMP, at higher concentrations (approx. $2.5 \cdot 10^{-7}$–$5 \cdot 10^{-6}$ M), stimulated the enzyme activity maximally, i.e. to the same extent as did cyclic GMP (approx. $1.0 \cdot 10^{-4}$ M) (Figs 3 and 4).

Higher concentrations of cyclic AMP decreased the enzyme activity. The combined effect of cyclic AMP and GMP on the enzyme activity is shown in Table I.

Cyclic GMP had no effect on the enzyme activity at an optimal concentration ($5 \cdot 10^{-6}$ M) of cyclic AMP. When a suboptimal concentration ($5 \cdot 10^{-8}$ M) of cyclic

TABLE I

EFFECT OF COMBINATION OF CYCLIC AMP AND GMP ON PROTEIN KINASE ACTIVITIES

Incubation conditions were described in the text, except for the variation in the cyclic nucleotides. All values must be compared with 3.5 ± 0.1 units/mg protein × 10^{-2} of the activity which occurred in the absence of added cyclic nucleotide.

Cortex				Medulla			
Cyclic nucleotides		Cyclic AMP (M)		Cyclic nucleotides		Cyclic AMP (M)	
		$5.0 \cdot 10^{-8}$	$5.0 \cdot 10^{-6}$			$5.0 \cdot 10^{-8}$	$5.0 \cdot 10^{-6}$
Cyclic GMP (M)	0	(unit/mg protein × 10^{-2}) 9.9 ± 1.4*	15.6 ± 1.3	Cyclic GMP (M)	0	(unit/mg protein × 10^{-2}) 6.6 ± 1.6	10.2 ± 1.1
	$2.5 \cdot 10^{-6}$	15.8 ± 2.4	17.1 ± 1.5		$2.5 \cdot 10^{-6}$	10.1 ± 1.4	9.8 ± 1.6
	$1.0 \cdot 10^{-4}$	14.5 ± 0.5	15.4 ± 0.6		$1.0 \cdot 10^{-4}$	10.4 ± 0.6	11.0 ± 1.6
No cyclic AMP		Cyclic GMP (M)		No cyclic AMP		Cyclic GMP (M)	
		$2.5 \cdot 10^{-6}$	$1.0 \cdot 10^{-4}$			$2.5 \cdot 10^{-6}$	$1.0 \cdot 10^{-4}$
	0	(unit/mg protein × 10^{-2}) 7.4 ± 1.4	14.3 ± 0.8		0	(unit/mg protein × 10^{-2}) 8.4 ± 2.8	12.3 ± 2.5

* Mean ±S.E. from 4 samples.

TABLE II

ESTIMATION OF THE BINDING AFFINITY AND ACTIVITY OF PROTEIN KINASE PREPARATION TO CYCLIC AMP AND GMP AT pH 6.5

Duplicate assays were performed by incubating 0.05 M potassium phosphate buffer, pH 6.5, cyclic [^{3}H]AMP or cyclic [^{3}H]GMP as indicated, with 190–220 μg of the protein kinase preparation in a total volume of 0.3 ml.

Kinase preparation	Affinity K_a, (× 10^{-8} M)		Activity (pmoles/mg protein)
Cortex	Cyclic AMP	0.8	5.6
	Cyclic GMP	11.0	0.7
Medulla	Cyclic AMP	3.2	1.2
	Cyclic GMP	6.9	0.8

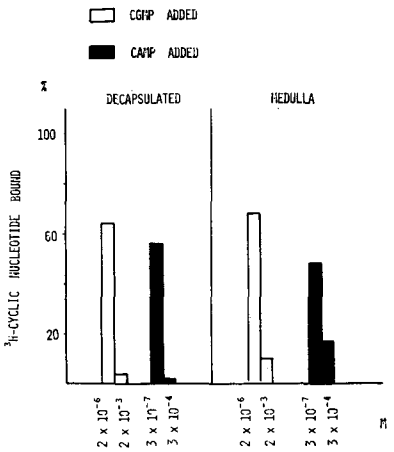

Fig. 6. Effects of various concentrations of nucleotides on the binding of cyclic [^{3}H]AMP or cyclic[^{3}H]GMP. The reaction mixture contained a 0.05 M potassium phosphate buffer, pH 6.5, $5 \cdot 10^{-8}$ M cyclic [^{3}H]AMP or $3 \cdot 10^{-7}$ M cyclic [^{3}H]GMP, 220 mg of the protein preparation, and unlabeled cyclic AMP or GMP as indicated.

AMP was added, the addition of cyclic GMP increased the activity in an additive fashion. The binding affinity of cyclic[^{3}H]AMP and [^{3}H]GMP to the protein from the cortex and the medulla is shown in Table II.

The binding activity of cyclic AMP was higher for the cortex protein than for the medullary protein. The binding activity of cyclic GMP, however, was similar for both proteins (Table II). The binding interaction of cyclic AMP and GMP is shown in Fig. 6. The binding of cyclic [^{3}H]AMP or cyclic [^{3}H]GMP was reduced by 48 and 65% at optimal binding concentrations of the other unlabeled cyclic nucleotides ($2 \cdot 10^{-6}$ M for cyclic GMP and $3 \cdot 10^{-7}$ M for cyclic AMP).

DISCUSSION

Cyclic nucleotide (cyclic AMP and GMP)-binding proteins and cyclic nucleotide-dependent protein kinases in the cortex and the medulla from bovine adrenal glands were studied.

Fractions of cyclic AMP- and cyclic GMP-binding proteins and of cyclic AMP- and cyclic GMP-dependent protein kinases appeared to be identical in DEAE chromatography (Figs 1 and 2). A protein kinase, preferentially activated by cyclic GMP has been isolated from the lobster muscle [9] and tissues from several other species and phyla [10, 11]. In this experiment on the bovine adrenal gland, cyclic GMP-dependent protein kinase which is more sensitive to cyclic GMP than to cyclic AMP was not separated by DEAE chromatography. Cyclic nucleotides stimulated the protein kinase of the cortex more than that of the medulla (Fig. 5), apparently due to the binding activity to the protein (Table II). This indicated that the mechanism of activation by cyclic nucleotides of the catalytic subunit of protein kinase would be by dissociation of the (inhibitory) R-protein subunit binding to the cyclic nucleotide [5, 12–17].

The discrepancy between values of binding affinity (K_a) and K_m of the protein

kinase for the cyclic nucleotide could be attributed to the differences in experimental conditions between the binding and the enzyme assay. Miyamoto et al. [18] found that dissociation of the protein kinase from bovine brain into subunits in the presence of histone, resulted in the conversion of the enzyme activity from a cyclic AMP-dependent to a cyclic AMP-independent form. Reaction of the cyclic AMP-independent protein kinase in the enzyme preparation would influence the action of the cyclic AMP-dependent enzyme. The cortical cyclic AMP-dependent protein kinase was more sensitive to cyclic AMP than was the medullary enzyme, which may be relevant to previous findings [2, 19] that adrenocorticotropin or stress produced a great increase in the cyclic AMP levels of the cortex, while no changes were observed in the medulla. The recent finding [20], that in rat cerebellum, where there is a cyclic GMP-dependent protein kinase, differences in stimulation of the enzyme by cyclic nucleotides are dependent on phosphate concentration and on the pH of the incubation medium, emphasizes the importance of incubation conditions when comparing protein kinase activities. Activation of the cortical cyclic AMP-dependent protein kinase by varying concentrations of cyclic AMP and GMP, showed that cyclic AMP was about 35 times more effective than cyclic GMP (Fig. 3). The medullary enzyme, however, responded similarly to both cyclic AMP and GMP (Fig. 4). Yamamura et al. [21] have reported that protein kinases and regulatory subunits from rat liver and rabbit skeletal muscle were crosswise reactive. Recently, multiple forms of hepatic cyclic AMP-dependent protein kinase were found, which are catalytically identical enzymes, but which differ in their associated regulatory proteins [16, 17].

Differences in cortical and medullary cyclic AMP-dependent protein kinases could be attributed to differences in regulatory proteins to which cyclic AMP binds in an allosteric manner. The combined use of cyclic AMP and GMP in the binding experiment (Fig. 6) and the protein kinase assay (Table I), indicated that cyclic GMP binds to the cyclic AMP-binding site of the protein kinase.

Since the activity of the cortex cyclic AMP-dependent protein kinases is very high, the influence of cyclic GMP could be ruled out. In the medulla, however, the role of cyclic GMP should be discussed together with that of cyclic AMP.

Since the discovery of the cyclic GMP-dependent protein kinase from the lobster muscle [9], it has been suggested that cyclic AMP and GMP are under separate regulatory control in a initial activation of the protein kinase. Kuo et al. [22] reported that some rat tissues contain a high concentration of cyclic GMP.

Recent papers [22–24] also reported that acetylcholine stimulates cyclic GMP production in a different manner from cyclic AMP production. Our recent experiments (Shima, S., Kawashima, Y. and Nakao, T., unpublished) in which acetylcholine, at doses sufficient to release catecholamines from bovine adrenal medulla slices, increased cyclic GMP levels, suggests the biological significance of cyclic GMP in the adrenal medulla. It is hoped that the role of cyclic GMP in the adrenal medulla will be clarified.

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