STUDIES ON CYCLIC NUCLEOTIDES IN THE ADRENAL GLAND
II. PROPERTIES OF CYCLIC AMP DEPENDENT PROTEIN
KINASES IN THE ADRENAL GLAND

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Abstract—Studies on the zonal function of CAMP-binding protein (regulatory pro-
tein) and CAMP-dependent protein kinases in bovine adrenal glands have been made. Partially purified CAMP-binding protein from bovine adrenal gland divided into three zones, the capsular (zona glomerulosa), the decapsulated (zona fasciculata and reticularis) and the medulla gland was found in microsomal and cytosol fractions. The affinity and activity of the protein binding to CAMP from the cortex (the capsular, $K_a$, $4.7 \times 10^{-9}$ M; activity, 4.6 p mole/mg protein, and the decapsulated, $K_a$, $4.2 \times 10^{-9}$ M; activity, 4.7 p mole/mg protein) was higher than from the medulla ($K_a$, $17 \times 10^{-9}$ M; activity, 2.2 p mole/mg protein). Two CAMP-dependent protein kinases, partly associated with CAMP-binding regulatory protein were partially purified from the cortex (the capsular and the decapsulated gland) and the medulla gland. The $K_m$ ($8.6 \times 10^{-8}$ M) for CAMP of protein kinases from the cortex (the decapsulated gland) was lower than the $K_m$ ($1.7 \times 10^{-7}$ M) from the medulla gland. Higher activity of CAMP-dependent protein kinases from the cortex than the medulla was discussed on the basis of differences in their regulatory proteins to which CAMP binds in an allosteric manner.

It is well known that the action of hormone is mediated by adenosine cyclic 3', 5'-monophosphate (CAMP) (1-3), which in turn activates various biological mechanisms, involving intracellular protein phosphorylation (4-5). Recent papers (6-9) proposed that the action of CAMP is involved in binding to a regulatory subunit (a regulatory protein) of protein kinase for dissociation of the holoenzyme into a binding subunit and a catalytic subunit, which results in deinhibition and initiation of the catalytic activity. In this laboratory studies on the functional zonation of the adrenal gland on differences in steroidogenic responses to ACTH have been made (10-13). The present paper was designated to study the mechanism of CAMP action on cyclic nucleotides-binding proteins and protein kinases in three zones of the adrenal gland, the capsular (zona glomerulosa), the decapsulated (zona fasciculata-reticularis) and the medulla.

MATERIALS 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 medulla zone. Cyclic AMP (CAMP) and dibutyril cyclic
AMP (DB-CAMP) were generously donated by Seishin Seiyaku, Co. Ltd., Tokyo, Japan. 

$^3$H-CAMP with a specific activity 20.7 Ci/m mole and adenosine 5'-triphosphate-$\gamma$-$^3$P (ATP-$\gamma$-$^3$P) with specific activities 1.0 to 2.8 Ci/m mole, were purchased from the Radiochemical Centre, Amersham, England. Histone from calf thymus (type II) and DEAE-cellulose were from Sigma Chemical Company, St. Louis., Mo.

Protein kinases and cyclic nucleotides-binding protein from three zones were partially purified by modification of the method of Kumon et al (7). 15 g of tissue were homogenized with 5 volumes 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 g for 15 min followed by a 100,000 g centrifugation of the supernatant for 120 min. An ammonium sulfate precipitation and adsorption to calcium phosphate gel was performed as described (7). The eluate of 0.2 M potassium phosphate buffer from calcium phosphate gel was again dialyzed against 10 mM Tris-HCl buffer, pH 7.5, containing 10% glycerol and 6 mM 2-mercaptoethanol (TMG buffer). The enzyme solution was applied to a column of DEAE-

![Graph](image-url)

**Fig. 1.** DEAE-cellulose column chromatography of protein kinase and CAMP-binding protein. Assay conditions of protein kinase activities in the presence (●—●) or absence (○—○) of $5 \times 10^{-4}$M CAMP, and CAMP-binding protein (x-----x) are as described in the text.
cellulose (2 x 10 cm) equilibrated with TMG buffer. Elution was carried out with a linear concentration gradient from 0.05 M to 0.5 M NaCl in TMG buffer.

An assay of binding of $^3$H-CAMP to the protein was followed by the method of Walton and Garren (14). The reaction mixture (0.30 ml) contained 50 mM potassium phosphate buffer (pH 6.5), 8 mM theophylline, $5 \times 10^{-5}$ M $^3$H-CAMP, and a protein preparation was incubated for 1 hr at 0°C. The reaction was terminated by dilution with 1 ml of ice-cold 25 mM Tris-HCl buffer at pH 8.0 containing 10 mM MgCl$_2$.

A millipore filter (HA 0.45 μ, 25 mm) was used to separate the free from $^3$H-CAMP bound to protein.

An assay of the protein kinase activity was based on the method of Miyamoto et al. (15). The incubation medium contained 10 μ moles of sodium glycerol phosphate buffer, pH 6.0; 0.6 mg of histone; 1.06 μ moles of ATP-$\gamma$-32P; 2 μ moles of magnesium acetate; 0.4 μ mole of theophylline; 0.06 μ mole of ethylene glycol bis (β-amino-ethyl ether)-N, N'-tetra-acetic acid; with or without $5 \times 10^{-6}$ M of CAMP. The incubation was carried out at 30°C for 15 min, and the reaction was stopped by addition of 4 ml of ice-cold 7.5% 

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**Fig. 2.** Depression of binding induced by adding various amounts of unlabeled CAMP. Incubation conditions were as described in the text.
trichloro-acetic acid, then 0.2 ml of 0.63% bovine serum albumin was added. The precipitate washed 3 times with 5% trichloroacetic acid was assayed for radioactivity with a liquid scintillation spectrometer (Aloka, Tokyo, Japan). Protein determination was made by the method of Lowry et al. (16). One unit of the enzyme activity was expressed as the amount of the enzyme incorporating 1 p mole of $^{32}$P from ATP-$^{32}$P to the recovered protein during 15 min incubation.

RESULTS

DEAE-cellulose chromatographies from three zones are shown in Fig. 1. Two peaks of the CAMP-dependent protein kinases, partly associated with CAMP-binding protein were observed in a linear concentration gradient elution. The elution pattern of the CAMP-dependent protein kinases from the medulla gland differed from the two zones of the cortex, the capsular and the decapsulated gland.

Binding of CAMP to the protein both found in the microsomal and the soluble fraction from the capsular and the decapsulated gland (Fig. 2) appears to be a single type of noninteracting bind site (Fig. 3). The binding affinity of the protein to CAMP from the capsular with a binding constant $K_a$ of $4.7 \times 10^{-9}$ M, and the decapsulated gland with $K_a$ of $4.2 \times 10^{-9}$ M, was higher than $K_a$ of $17.0 \times 10^{-9}$ M from the medulla gland (Figs. 1 and 4).

Activity of the protein kinase as a function of time and of protein concentration is shown in Figs. 5, 6, and 7. Fig. 7 demonstrates that the enzyme activity from the medulla gland was lower than the capsular (Fig. 5) and the decapsulated gland (Fig. 6). A com-

![Fig. 3. Interaction of $^3$H-CAMP with binding protein as a function of total concentration of the nucleotide added.](Image)

Duplicate assays were performed by incubating 50 mM potassium phosphate buffer, pH 6.5. Incubation conditions were as described in the text.
Fig. 4. Estimation of CAMP binding constant at pH 6.5, of the capsular (○—○), the decapsulated (●—●) and the medulla (×—×). Incubation conditions are as described in the text.

Fig. 5. Effect of $5 \times 10^{-6}$ M CAMP on protein kinase activities of the decapsulated gland as a function of time and of enzyme concentration in the presence (●—●) or absence (○—○) of CAMP.

Comparison of the protein kinase activity between the cortex (the decapsulated gland) and the medulla gland in various concentrations of CAMP added is shown in Fig. 8. The $K_m$ ($8.6 \times 10^{-5}$ M) for CAMP of the enzyme from the decapsulated gland was lower than
the medulla (Km 1.7 × 10⁻⁷ M). Effects of DB-CAMP on the enzyme activity were almost negligible with the exception of a slight increase in the high concentration of DB-CAMP (Fig. 8).

It is of interest that the enzyme activity was enhanced with concomitant increase in CAMP concentration and decreased in a concentration higher than 5 × 10⁻⁶ M.
FIG. 8. Comparison of protein kinase activities responding to various concentrations of CAMP and dibutyryl CAMP (△—△) between the decapsulated (●—●) and the medulla (○—○). Assay conditions were as described in the text. Effects of dibutyryl CAMP were on the enzyme from the decapsulated.

DISCUSSION

The zonal function of CAMP binding and protein kinase in three zones, the capsular, the decapsulated and the medulla from bovine adrenal glands was studied.

Previous experiments on rat adrenal glands (12, 17, 28) revealed that the adenyl cyclase activities of the capsular and the decapsulated were the same, regardless of great differences in steroidogenic responses to ACTH between the two zones. In the present paper, the activity of CAMP binding and protein kinases stimulated by CAMP was also similar in the capsular and the decapsulated zone.

These results suggest that all amounts of CAMP induced by ACTH and the subsequent increase in the activity of protein kinases could not be responsible for the rate of steroidogenesis in these two zones. The activity of CAMP binding and protein kinases in the medulla gland differed from the cortex gland (Figs. 1, 2, 4 and 8). Treatments with ACTH in vitro (12, 17, 18) and immobilization stress (17, 18) to rats produced a great increase in adenyl cyclase activity in the adrenal cortex (the capsular and the decapsulated gland) without any change in the medulla.

Higher affinity (Ka, 4.2 to 4.7 x 10^-9 M) and activity (4.6 to 4.7 p mole/mg protein) of CAMP binding to the regulatory protein in the cortex than the medulla (Ka, 17 x 10^-9 M; activity, 2.1 p mole/mg protein) (Fig. 4) may be closely related to higher activity of protein kinases in the cortex than in the medulla. In Fig. 8, a lower Km of protein kinases for CAMP in the decapsulated rather than in the medulla indicates that the enzyme in the
adrenal cortex is more dependent on CAMP than the medulla.

CAMP dependent protein kinases found in a number of mammalian tissues such as rabbit reticulocytes (8), bovine adrenal gland (17–19), bovine brain (15), rabbit skeletal muscle (9, 20), mouse mammary gland (21), rat adipose tissue (22), and rat liver (7, 23, 24) could be released and activated by CAMP bound to their regulatory subunit proteins. Yamamura et al. (25) have reported that protein kinases and regulatory proteins from rat liver and rabbit skeletal muscle were crosswise reactive. Recently, the multiple forms of hepatic CAMP-dependent protein kinase defined at least as a catalytically identical enzyme were found (26, 27) and may be different from each other in associated regulatory proteins. Differences in protein kinases between the cortex and the medulla presented here can be attributed to differences in regulatory proteins to which CAMP binds in an allosteric manner.

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