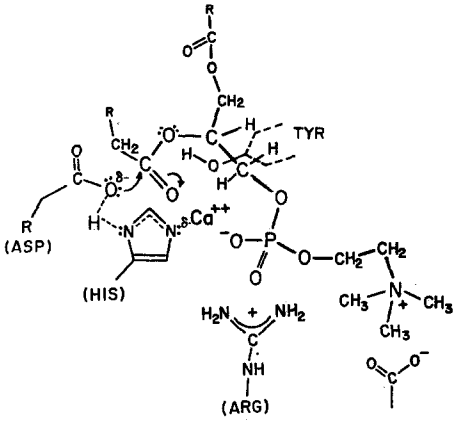

Figure 2. An active-site model for the pancreatic phospholipase A_2.

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In "Demonstration of ACTH-Sensitive Particulate Guanylate Cyclase in Adrenocortical Carcinoma," by Ponnal Nambi and Rameshwar K. Sharma, pp. 508-514, through a typographical error the Figs. 3 and 4 that appear on pages 511 and 512, respectively, are incorrect. For the readers' convenience, the entire article with the correct Figs. 3 and 4 is reprinted beginning on the next page.

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DEMONSTRATION OF ACTH–SENSITIVE PARTICULATE GUANYLATE CYCLASE IN ADRENOCORTICAL CARCINOMA

PONNAL NAMBI AND RAMESHWAR K. SHARMA

Department of Biochemistry
Laboratory of Basic Hormone Research
University of Tennessee
Center for the Health Sciences
Memphis, Tennessee 38163

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SUMMARY

The guanylate cyclase activity was detectable both in particulate and soluble fractions from adrenocortical carcinoma. The guanylate cyclase in the particulate fraction was stimulated by low concentration of ACTH, 7×10^{-11}M, but not by sodium nitroprusside or ascorbic acid. The presence of calcium was found to be essential for this ACTH–stimulation of guanylate cyclase. When calcium was omitted from the assay or the calcium chelator EGTA was added to the incubation medium, no stimulation by ACTH was observed. Calcium alone could not replace ACTH in activating the cyclase enzyme. The soluble guanylate cyclase was stimulated by sodium nitroprusside but not by ascorbic acid or ACTH. These data demonstrate the hormonally–sensitive particulate guanylate cyclase in a cell–free system.

INTRODUCTION

Despite the fact that ACTH in the adrenal cortex (1–3) and adrenocortical carcinoma cells (4), as well as several neurotransmitters and hormones in intact target tissues (5,6) cause an increment in the levels of cyclic GMP, previous attempts to demonstrate a hormone–sensitive guanylate cyclase in cell free preparations of any tissue have failed (6). This communication describes a specific ACTH–responsive particulate guanylate cyclase in adrenocortical carcinoma. In addition, we demonstrate that this cyclase is not stimulated by sodium nitroprusside and ascorbic acid, the two agents that have been shown to stimulate the enzyme in various other tissues (6).

The abbreviation used are: EGTA, ethylene glycol bis (β–aminoethyl ether) N,N,N′,N′–tetra acetic acid; Gpp(NH)p, 5′–guanylylimido diphosphate.

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MATERIALS AND METHODS

GTP, Gpp(NH)p, ATP, creatine phosphate, and creatine kinase were obtained from Sigma. ACTH was from USP Corticotropin Reference Standard, Bethesda, Maryland. All other reagents were analytical grade and were obtained commercially.

The adrenocortical carcinoma 494, a spontaneously occuring tumor discovered by Snell and Stewart (7) and maintained in our laboratory (8) was used as a source of cyclase enzymes. The particulate fractions were prepared following the procedure of Williams et al (9) with minor modifications. Fresh tumor tissue was cleaned of all fat and necrotic tissue, and then minced and homogenized in 6 volumes of ice cold buffer (0.25 M sucrose, 1 mM $MgCl_2$, 5 mM Tris, pH 7.5) for 4 x 30 second periods using a Brinkmann Polytron at a setting of 6. After filtration through a double layer of gauze, the homogenate was centrifuged at 400 x g for 10 min. and the supernatant was centrifuged at 100,000 x g for 1 hour. The final pellet resuspended in incubation buffer was used as a source of particulate cyclase and the supernatant as a source of soluble cyclase.

Guanylate cyclase was assayed following the procedure of Garbers and Murad (10), using unlabled GTP and the determination of cyclic GMP was done by radioimmunoassay (11). The sensitivity of the method was increased by acetylation of samples prior to radioimmunoassay (12). Generally, the incubation tubes contained 50 μl of a freshly prepared assay mixture (40 μl of 25 mM theophylline, 5 μl of 1 M Tris–HCl pH 7.6, 5 μl of creatine phosphate–creatine kinase mixture) to which was added 30 μl of distilled water or various test reagents and 10 μl of enzyme solution. The reaction, in a final volume of 100 μl, was initiated by the addition of 10 μl of substrate containing 10 mM GTP and 40 mM $MnCl_2$. Incubation (37°C, 10 min) was terminated by the addition of 0.90 ml of 50 mM sodium acetate buffer, pH 4.0 followed by heating the mixture for 3 min. in a boiling water bath. Supernatant fractions obtained by centrifugation at 1500 rpm for 20 min. were used for radioimmunoassay. Samples incubated without enzyme or with heated enzyme served as controls. All assays were performed in duplicate and were repeated at least five times and the data presented are mean values from representative experiments. Protein was determined by the Bradford method with the use of bovine serum albumin as a standard (13).

RESULTS AND DISCUSSION

Figure 1 shows that 7×10^{-11}M ACTH markedly stimulates adrenocortical carcinoma particulate guanylate cyclase. The comparable concentration of the hormone does not stimulate the adenylate cyclase. In contrast to the ACTH–dependent activation of particulate guanylate cyclase, the soluble guanylate cyclase is unaffected by the hormone. These data indicate that the carcinoma guanylate cyclase exists in two forms, particulate and soluble, and only the particulate enzyme is hormonally–dependent.

It is noteworthy that only the low concentration of ACTH activates the particulate guanylate cyclase. Concentrations in excess of 7×10^{-11}M cause a decline in the peak level of the nucleotide. Similar profile of the rise of cyclic GMP in response to ACTH has been reported earlier in intact isolated adrenal (1) and adrenocortical carcinoma cells (2). At this time we do not know the reason for the decline of peak guanylate cyclase in response to the higher concentrations of ACTH. With intact isolated adrenocortical carcinoma cells, we have previously provided evidence that the ACTH–stimulated rise of cyclic GMP activates cyclic GMP–phosphodiesterase resulting in the fall of cyclic GMP levels (14). It is possible that a similar mechanism operates in the cell–free system.

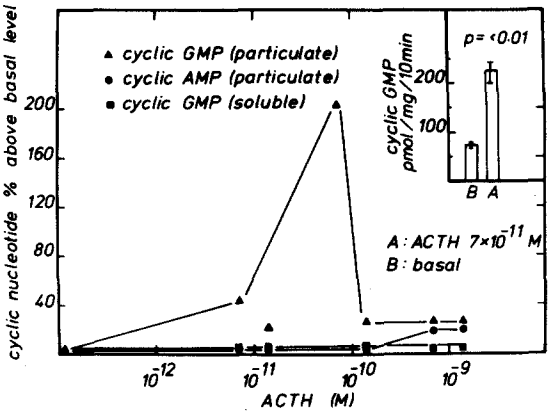

Fig. 1 Effects of ACTH on guanylate and adenylate cyclases of the adrenocortical carcinoma. Incubation mixtures for guanylate cyclase assay contained 1 mM GTP, 4 mM $MnCl_2$, 10 mM theophylline, regenerating system (creatine kinase 20 μg, creating phosphate 15 mM) 1 mM $CaCl_2$, 10 μl of 100,000 x g pellet fraction (30–60 μg protein) and various concentrations of ACTH. Results are expressed as % above basal level of both nucleotides. The incubation mixtures for adenylate cyclase assay was the same as that of guanylate cyclase except 1 mM ATP and 2 mM $MgCl_2$ were used instead of GTP and $MnCl_2$. The basal level of guanylate cyclase was 75 pmol/mg protein/10 min. while that of adenylate cyclase was 73 pmol/mg protein/10 min. The experiments were done in duplicate and repeated at least 5 times. Although the data depicted are from one experiment, all experiments showed the same pattern.

Inset: The stimulation of particulate guanylate cyclase in response to 7 x 10^{-11}M ACTH. The values of p were calculated from the data obtained from 5 experiments with a total of 10 observations.

Investigations from several laboratories have indicated that guanylate cyclase from various tissue sources is stimulated by sodium nitroprusside and in some cases by ascorbic acid (6). Data depicted in Figure 2 show that these agents do not stimulate adrenocortical carcinoma particulate guanylate cyclase. On the other hand, soluble guanylate cyclase is activated by sodium nitroprusside in a concentration–response manner, but ascorbic acid has no effect on this enzyme activity. These data further attest to the specificity of the ACTH–dependent guanylate cyclase.

Figure 3 shows that lmM Ca^{2+} in the presence of 4 x 10^{-11}M ACTH maximally activates the particulate adrenocortical carcinoma guanylate cyclase, but the hormonally–dependent activation of the cyclase is lost when Ca^{2+} is omitted from the incubation medium. This indicates that the requirement of Ca^{2+} is absoulte in ACTH–induced guanylate cyclase. The ACTH–dependent cyclase activation is directly proportional to calcium concentrations ranging from 0 to 1 mM Ca^{2+}. Higher concentrations of Ca^{2+}, however, inhibit guanylate cyclase. It is noteworthy that Ca^{2+} alone is unable to activate guanylate cyclase. This indicates that the presence of calcium is

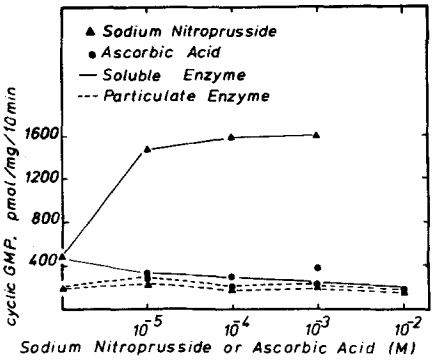

Fig. 2 Effects of various concentrations of sodium nitroprusside and ascorbic acid on particulate and soluble guanylate cyclases of adrenocortical carcinoma. Various concentrations of the compounds were added directly to the assay and the conditions of the experiments were the same as described in Figure 1.

obligatory in the ACTH–activated guanylate cyclase but calcium alone cannot stimulate guanylate cyclase. We have demonstrated a similar role of calcium in ACTH–induced steroidogenesis that is mediated by the specific increment of cyclic GMP in intact isolated fasciculata cells (15). We propose that Ca^{2+} is one of the coupling factors that is essential for the hormonally–dependent guanylate cyclase.

It is well established that adenylate cyclase from mammalian tissues is stimulated by fluoride and Gpp(NH)p (16–18). Similarly, adrenocortical cardinoma adenylate cyclase is also stimulated by fluoride and Gpp(NH)p. However, in contrast, fluoride does not affect

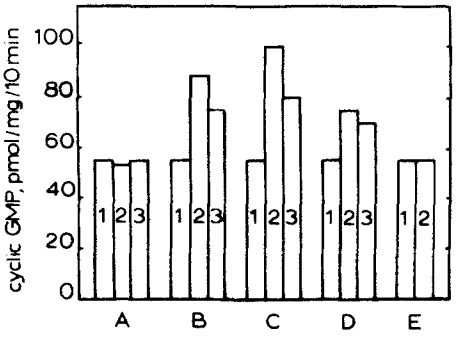

Fig. 3 Effect of Ca^{2+} on particulate guanylate cyclase. The conditions of the experiments were the same as those described in Figure 1. No Ca^{2+} in the assay (A); 0.1 mM Ca^{2+} (B); 1.0 mM Ca^{2+} (C); 3.0 mM Ca^{2+} (D); 0.5 mM EGTA (E). Basal (1); ACTH 4×10^{-12} M (2); ACTH 4×10^{-11}M (3).

TABLE I

Effect of Gpp(NH)p and sodium fluoride on particulate guanylate and adenylate cyclase activities.

Indicated concentrations of Gpp(NH)p and sodium fluoride were added to the incubation mixtures and the assays were conducted after 5 min. preincubation at 37°C.

CONDITION	GUANYLATE CYCLASE	ADENYLATE CYCLASE
	cyclic nucleotide pmol/mg protein/10 min.	
Basal	88	70
Gpp(NH)p (1mM)	45	350
Sodium Fluoride (5 mM)	89	360
Gpp(NH)p (1mM)*	20	

*Gpp(NH)p was used as the substrate for guanylate cyclase instead of GTP and the regenerating system was omitted from the assay.

the adrenocortical particulate guanylate cyclase and Gpp(NH)p strongly inhibits it (Table 1). The inhibition by the guanine nucleotide might be due to its competition with the substrate GTP. When Gpp(NH)p is used as a substrate for the cyclase reaction, it is only 20% as effective as GTP. ATP strongly inhibits the guanylate cyclase activity of both particulate and soluble enzymes (Figure 4). These results indicate that the particulate fraction contains

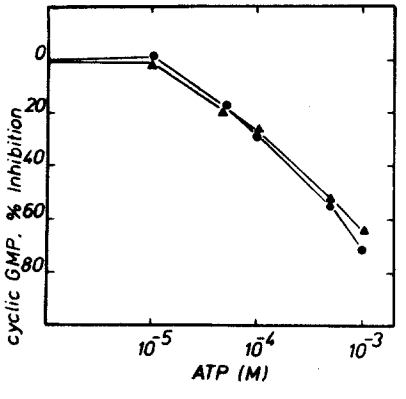

Fig. 4 Effect of various concentrations of ATP on particulate (▲) and soluble (●) guanylate cyclases of adrenocortical carcinoma. Various concentrations of ATP were added directly to the assay and the conditions of the experiment were the same as described in Figure 1.

adenylate cyclase which responds to its usual activators, but is unresponsive to ACTH. These data further indicate that, as expected, the specific substrate for the guanylate cyclase is GTP.

Previous studies with intact fascicula cells provide evidence supporting the important mediatory role of cyclic GMP and calcium in adrenal steroidogenesis (15). Nonetheless, this role of the cyclic nucleotide remained partly speculative due to the lack of direct demonstration of the ACTH–dependent guanylate cyclase in cell–free preparations. The data presented here clearly demonstrate such a hormonally–dependent guanylate cyclase in the carcinoma cells. This finding therefore bridges a gap that previously existed in the chain of events leading from the binding of the hormone to its receptor and the biological response.

ACKNOWLEDGEMENTS

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