METABOLIC REGULATION OF STEROIDOGENESIS IN ISOLATED ADRENOCORTICAL CARCINOMA CELLS. ACTH REGULATION OF GUANOSINE CYCLIC 3':5' - MONOPHOSPHATE LEVELS

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Summary: In the adrenocortical carcinoma cell, 2.5 to 10 μunits of ACTH do not raise the adenosine cyclic 3':5' - monophosphate (cyclic AMP) level, protein kinase activity, and steroidogenesis, whereas there is a sharp increase in the level of guanosine cyclic 3':5' - monophosphate (cyclic GMP). This change in the level of cyclic GMP indicates an active guanylate cyclase system since carcinoma cells are devoid of detectable cyclic 3':5' - nucleotide monophosphate phosphodiesterase activity. In a normal adrenal cell these concentrations of the hormone markedly increase cyclic GMP level with the concomitant increase in protein kinase activity and corticosterone formation. These results indicate therefore that the lack of regulation of corticosterone synthesis by cyclic GMP in adrenocortical carcinoma cell may be due to the abnormal cyclic GMP-dependent protein kinase. That the tumor ACTH - sensitive guanylate cyclase system is not under the transcriptional or translational control is indicated by the findings that actinomycin D and cycloheximide do not inhibit the ACTH - activated level of cyclic GMP.

Introduction

In contrast to normal isolated cells (1-3), which are markedly stimulated by microunit concentrations of ACTH to form corticosterone, tissue slices (4) or cells (5) isolated from adrenocortical carcinoma 494 (6) do not respond to this hormone in the activation of steroidogenesis. An investigation to answer this question has revealed various biochemical lesions both before and after the events leading to the cleavage of the cholesterol side-chain. These metabolic lesions are responsible for the altered but unique ACTH-controlled system in these cells. These defects have been observed at the level of the plasma membrane (7, 8), adenylate cyclase system (9, 10), phosphodiesterase activity (11), and in the steroid biosynthetic transformations of (20S)-20-hydroxy-cholesterol (12, 13), pregnenolone, progesterone and deoxycorticosterone to corti-

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costerone (14). Specific studies designed to analyze the tumor adenylate cyclase in the intact cell have indicated that 10 to 50 μunits of ACTH, in contrast to the normal adrenal cell, do not raise the level of cyclic AMP thus indicating a lesion in the tumor adenylate cyclase system (15). Accelerated hydrolysis of cyclic AMP in the tumor cannot account for the lack of cyclic AMP stimulation since the adrenocortical carcinoma cells used in these studies have no detectable cyclic 3':5' - nucleotide monophosphate phosphodiesterase activity (16). Nonetheless, two questions remained to be answered. First is that why exogenous steroidogenic concentrations,of cyclic AMP, in contrast to the normal cells (1, 2), do not stimulate corticosterone synthesis and the second is that whether submaximal steroidogenic concentration of ACTH (< 10 μunits) raises the levels of cyclic GMP? The latter finding would reflect the presence or absence of an active guanylate cyclase since the isolated adrenocortical carcinoma cells are devoid of detectable cyclic 3':5' - nucleotide monophosphate phosphodiesterase activity (16).

Studies designed to answer the first question have indicated that one of the reasons for the lack of cyclic AMP-stimulated corticosterone synthesis may be due to the presence of the defective cyclic AMP-dependent protein kinase system. Cyclic AMP-binding protein kinase enzyme has been partially purified (17). This enzyme specifically binds cyclic AMP but fails to phosphorylate exogenous histone. It has been postulated that the lack of the cyclic AMP-dependent kinase activity of this enzyme may be responsible for the loss of cyclic AMP-regulated corticosterone synthesis in adrenocortical carcinoma cell (17). The present investigation was designed to investigate the second question i.e. to analyze the guanylate cyclase system in adrenocortical carcinoma cells and then relate this information to the abnormal ACTH control as mediated by cyclic GMP.

Materials and Methods

The isolated adrenocortical carcinoma 494 cells were prepared by trypsin digestion (5). The method of incubation with ACTH and other agents has been already described (5, 14). In general for each isolated adrenocortical carcinoma cell preparation, 1.3 g of tumor tissues were used and the cells, representing 30 to 35 mg of adrenal tumor (approximately 2×10^6 cells), were resuspended in 0.8 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 4% albumin and 0.2% glucose.

The incubation experiments, in which corticosterone, cyclic AMP, cyclic GMP and protein kinase activity were measured simultaneously were conducted in sextuplicates; two of the samples were used for the determination of corticosterone, two for the measurement of cyclic AMP and cyclic GMP, and two for the assay of phosphorylation. The other experiments, in which only cyclic AMP and cyclic GMP were determined, were done in duplicate.

Extraction of cyclic AMP and cyclic GMP was performed as described previously (18). The assay of cyclic AMP was then accomplished by the method of Gilman (19) using the cyclic AMP-binding protein isolated from bovine kidney (20). The cyclic GMP was determined by the modified method (21) of Shibuya et al. (22) which utilizes the highly specific binding property of E. coli polypeptide chain elongation factor Tu (EF-Tu) with GDP. Cyclic GMP was estimated from the radioactivity of [β-^{32}P]GDP bound with crude E. coli fraction prepared as previously described (21). Endogenous protein kinase activity was determined as previously described (23). ACTH, with activity of 1.5 IU/ampule was purchased from United States Pharmacopeia. All other chemicals were reagent grade and were obtained commercially.

Results and Discussion

In order to determine the correlation between the endogenous levels of cyclic GMP formed in response to the varying concentrations of ACTH, isolated adrenocortical carcinoma cells were incubated with a series of concentrations of ACTH and the levels of corticosterone, cyclic GMP and cyclic AMP and protein kinase activity were measured (Fig. 1). The results show that in the intact isolated adrenocortical carcinoma cells, ACTH up to the concentration of 50 μunits does not cause the rise in cyclic AMP level whereas it raises the cyclic GMP level without a corresponding increase in corticosterone formation and protein kinase activity. In an isolated adrenal cell, less than 10 μunits of ACTH raises the level of cyclic GMP (18, 21, 23) with the concomitant increase in protein kinase activity followed by the activation of steroidogenesis. ACTH in a concentration range of 10 to 100 μunits raises the level of cyclic AMP

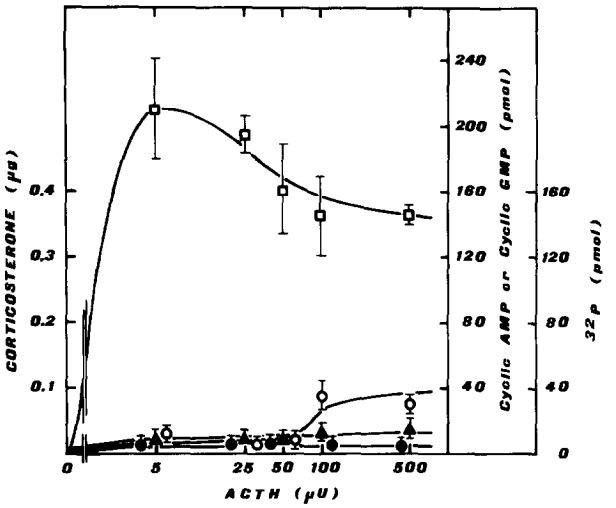

Figure 1. Concentration response curve for production of cyclic AMP (0--0), cyclic GMP (□-□), corticosterone (●-●), and phosphorylation (▲-▲) in isolated adrenocortical carcinoma cells incubated for 10 minutes in presence of 0 to 500 µU of ACTH. Incubation system: 2 x 10^6 isolated adrenocortical carcinoma cells suspended in 0.8 ml; reagents were dissolved in 0.2 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 4% albumin and 0.2% glucose, or protein kinase buffer. Total volume of first incubation, 1 ml and of second incubation, 0.5 ml. Results are expressed as the mean values (±S.D.) of 6 separate determinations from 3 different experiments. Basal values have been substracted from the experimental results (average basal value for corticosterone, 0.05 µg, for phosphorylation, 186 pmoles, for cyclic AMP, 34 pmoles, and for cyclic GMP, 78 pmoles).

and the process of steroidogenesis is accompanied by the stimulation of protein kinase (18, 21, 22). The present results obtained with the tumors therefore indicate that the tumor possesses a hormonally dependent guanylate cyclase system but is devoid of an active adenylate cyclase system. Maximal steroidogenic concentrations of ACTH raise the level of cyclic AMP but these levels are only 20 percent of those obtained for cyclic GMP. Previously (9, 10) it has been shown that pharmacological concentrations of ACTH (460 milliunits) activate tumor homogenate and particulate fraction adenylate cyclase activity. No stimulation by ACTH

Table 1

Effect of actinomycin D and cycloheximide on ACTH-induced cyclic GMP synthesis in adrenocortical carcinoma cells.

Incubation system: Isolated adrenal cell suspension, 0.8 ml; reagents dissolved in 0.2 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 4% albumin and 0.2% glucose. Total volume of incubation, 1 ml. Results are expressed as the mean values (±S.D.) of 6 separate determinations from 3 different experiments. Basal values have been substracted from the experimental results (average basal value for cyclic GMP, 78 ± 8 pmoles/10 minutes).

	Cyclic GMP (pmoles/10 min)		
ACTH μunits	Control	+2μM Actinomycin D	+10μM Cycloheximide
5	210 ± 38	216 ± 17	247 ± 38
25	196 ± 12	172 ± 16	204 ± 18
50	162 ± 30	185 ± 21	156 ± 32
100	145 ± 26	168 ± 18	130 ± 27
500	146 ± 4	158 ± 33	131 ± 19

at concentrations close to physiological ones was found, however (10). These lower concentration markedly affect steroid metabolism in the intact isolated fasciculata cell (1). The physiological significance of these findings (9, 10) is therefore questionable.

ACTH-activated rise in cyclic GMP (table 1) or cyclic AMP (table 2) levels is not inhibited by actinomycin D or cycloheximide indicating that the activities of the tumor guanylate or adenylate cyclases are not under the transcriptional or translational control. In a normal fasciculata cell cycloheximide almost completely inhibits (1) and actinomycin partially blocks (23) the ACTH-induced steroidogenesis. It has been further demonstrated that the cycloheximide-and actinomycin-sensitive control step in an isolated adrenal cell is before the events which lead to the

Table 2

Effect of actinomycin D and cycloheximide on ACTH-induced cyclic AMP synthesis in adrenocortical carcinoma cells.

The conditions of the experiment are identical to those described in Table 1. Results are expressed as the mean values (±S.D.) of 6 separate determinations from 3 different experiments. Basal values have been substracted from the experimental results (average basal value for cyclic AMP, 34 ± 3 pmoles/10 minutes).

	Cyclic AMP (pmoles/10 min)		
ACTH μunits	Control	+2μM Actinomycin D	+10μM Cycloheximide
5	6 ± 2	8 ± 4	9 ± 6
25	2 ± 1	7 ± 3	5 ± 4
50	8 ± 2	4 ± 1	2 ± 1
100	35 ± 12	29 ± 6	27 ± 4
500	28 ± 6	22 ± 3	29 ± 2

cleavage of cholesterol side-chain (24, 25). The defective step/s responsible for the lack of stimulation of steroidogenesis by the submaximal steroidogenic ACTH concentrations of normal cells therefore appears to be after the events which activate guanylate cyclase. Previously (5) it has been shown that the exogenous cyclic GMP also lacks the steroidogenic activity in the tumor cell. These results taken together indicate therefore the possibility of defective cyclic GMP-dependent protein kinase but to date no cyclic GMP-dependent protein kinase enzyme isolated from adrenal cortex has been reported, although there is a preliminary report (26) on the isolation of the cyclic GMP-binding protein. But if the tumor does indeed possess a defective cyclic GMP-dependent protein kinase system in addition to the defective cyclic AMP-dependent protein kinase system, then the abnormal regulation due to the lesions in protein

kinases might be a generality accompanied by malignancy in adrenocortical carcinoma. The result of this change in cyclic nucleotide metabolism might be useful to the survival of the malignant cell but the consequences to the malignant cell will be catastrophic.

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References

1. Kitabchi, A.E. and Sharma, R.K. (1971) Endocrinology, 88, 1109-1116.
2. Sharma, R.K., Hashimoto, K.E. and Kitabchi, A.E. (1972) Endocrinology, 91, 994-1003.
3. Sayers, G., Swallow, R.L. and Giordano, N.D. (1972) Endocrinology, 88, 1063-1068.
4. Ney, R.L., Hochella, N.J. and Grahme-Smith, D.G. (1969) J. Clin. Invest., 48, 1733-1739.
5. Sharma, R.K. and Hashimoto, K.E. (1972) Cancer Res., 32, 666-674.
6. Snell, K.C. and Stewart, H.L. (1959) J. Natl. Cancer Inst., 22, 1119-1132.
7. Sharma, R.K. (1974) FEBS Letters, 38, 197-201.
8. Sawhney, R.S. and Sharma, R.K. (1976) FEBS Letters, 70, 163-166.
9. Schorr, I. and Ney, R.L. (1971) J. Clin. Invest., 50, 1295-1300.
10. Brush, J.S., Sutliff, L.S. and Sharma, R.K. (1974) Cancer Res. 34, 1495-1502.
11. Sharma, R.K. (1972) Cancer Res., 32, 1734-1736.
12. Sharma R.K. and Brush, J.S. (1973) Arch. Biochem. Biophys., 156, 560-562.
13. Sharma, R.K. and Brush, J.S. (1974) Biochem. Biophys., Res. Commun., 56, 256-263.
14. Sharma, R.K. (1973) Eur. J. Biochem., 32, 506-512.
15. Sharma, R.K., Shanker, G. and Ahmed, N.K. (1977) Cancer Res., 37, 472-475.
16. Sharma, R.K. (1976) In: "Control Mechanisms in Cancer" (Criss, W.E., Ono, T. and Sabine, J.R. eds.) pp. 109-124, Raven Press, N.Y.
17. Sharma, R.K., Shanker, G., Ahrens, H. and Ahmed, N.K., Cancer Res., (In Press).
18. Sharma, R.K., Ahmed, N.K., Sutliff, L.S. and Brush, J.S. (1974) FEBS Letters 45, 107-110.

19. Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S., 67, 305-312.

20. Cheung, W.Y. and Patrick, S.M. (1974) Intern. J. Biochem., 5, 331-342.

21. Perchellet, J.P., Shanker, G. and Sharma, R.K., Science, (In Press).

22. Shibuya, M., Arai, K. and Kaziro, Y. (1975) Biochem. Biophys. Res. Commun., 62, 129-135.

23. Sharma, R.K., Ahmed, N.K. and Shanker, G. (1976) Eur. J. Biochem., 70, 427-433.

24. Sharma, R.K. (1973) J. Biol. Chem., 248, 5473-5476.

25. Sharma, R.K. (1974) Biochem. Biophys. Res. Commun., 59, 992-1004.

26. Gill, G.N. and Kanstein, C.B. (1975) Biochem. Biophys. Res. Commun., 63, 1113-1122.