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Research Article

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Abnormal Hormone Responses of an Adrenocortical Cancer Adenyl Cyclase

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ABSTRACT Properties of adenyl cyclase of normal adrenals and of a corticosterone-producing adrenal cancer of the rat have been compared. Enzyme activity was found in all particulate fractions of both tissues. The cyclase of the tumor as well as of the adrenals was stimulated by adrenocorticotropic hormone (ACTH) over similar concentration ranges. Unexpectedly, the tumor enzyme was also stimulated by epinephrine, norepinephrine, and thyroid-stimulating hormone (TSH). These hormones produced a dose-related effect over a concentration span that was comparable with that for ACTH. The tumor cyclase was not responsive to angiotensin II, vasopressin, glucagon, insulin, growth hormone, parathyroid hormone, and thyrocalcitonin. ACTH was the only hormonal preparation that stimulated normal adrenal cyclase. These findings are compatible either with the possibility that the adenyl cyclase receptor of the tumor has undergone structural alteration with a consequent loss of specificity for ACTH or with the possibility that the tumor possesses several cyclase regulatory receptors.

INTRODUCTION

Adrenocortical neoplasms that produce glucocorticoids or 17-ketosteroids (or both) are active in hormone production in the absence of adrenocorticotropic hormone (ACTH),[1] contrasting with the ACTH dependence of the normal adrenal cortex. Also, in contrast with the normal adrenals, the growth of the tumors is not dependent on the presence of ACTH. In the normal adrenal, ACTH stimulates the activity of adenyl cyclase, leading to increased conversion of adenosinetriphosphate (ATP) to cyclic adenosine 3′,5′-monophosphate (cyclic AMP) (1, 2). The cyclic nucleotide in turn brings about an accelerated rate of steroidogenesis (2, 3). These considerations have led us to examine the properties of adenyl cyclase in an adrenocortical carcinoma. As a readily and continuously available supply of tumor tissue, we have employed a corticosterone-producing transplantable rat adrenocortical carcinoma (4, 5). Previous studies have revealed that the tumor possesses adenyl cyclase activity capable of stimulation by ACTH (5). The studies described here have examined the subcellular distribution of adenyl cyclase in the tumor and have examined possible regulatory influences on the tumor enzyme. Unexpectedly, the tumor adenyl cyclase has been found to be responsive not only to ACTH, but also to certain other hormones as well.

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[1] *Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; cyclic AMP, cyclic adenosine 3′,5′-monophosphate; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone.

[2] Schorr, I.. and R. L. Ney. Unpublished observations.

METHODS

The tumor employed in these studies was rat adrenocortical carcinoma 494 originally found by Snell and Stewart (4). The tumor was maintained by transplantation in male Sprague-Dawley rats as previously described (5). Normal adrenals were obtained from male Sprague-Dawley rats weighing 160–180 g.

Tumor tissue and adrenals (50–400 mg/ml) were homogenized in a buffer containing 62.2 mM Tris (hydroxymethyl) aminomethane (HCl) and 15.5 mM theophylline at pH 7.4. Whole homogenates or fractions derived from them by centrifugation were used for adenyl cyclase assays. To obtain the fractions, the homogenate was centrifuged at 1000 *g* for 10 min. The sediment was collected and the supernatant centrifuged at 10,000 *g* for 10 min. This sediment was collected, and its supernatant was centrifuged at 105,000 *g* for 60 min. The sedimented particles were resuspended in the homogenizing medium, utilizing volumes adjusted to yield approximately equal protein concentrations in the different fractions in any given experiment. The tissue homogenates and centrifuged fractions were maintained at 4°C throughout these manipulations. Adenyl cyclase activity was determined by measuring the rate of conversion of α-ATP-^{32}P to ^{32}P-labeled cyclic AMP. Cyclic AMP uniformly labeled with tritium was added to allow correction for cyclic AMP breakdown during the incubation and for losses during subsequent purification steps. Homogenates or particles suspended in a volume of 40 μl

were added to the remainder of the components of the assay made up in a volume of 30 μl. The final concentrations of components in the assay mixture were 2 mM ATP, 3 mM $MgSO_4$, 2 mM 3',5'-cyclic AMP, 40 mM Tris, and 10 mM theophylline. 8 mM phosphoenol pyruvate and 0.3 U of pyruvate kinase per incubate were included as an ATP-generating system. Each assay tube contained α-ATP-^{32}P in amounts of 1–2 μCi in different experiments as well as 0.05 μCi cyclic AMP-^{3}H. The assays were carried out at pH 7.4 for 20 min in a water bath at 30°C with shaking. For each enzyme preparation assayed there was a blank. The blank was placed in a boiling water bath for 90 sec before the 20 min incubation, whereas the assay tube was boiled at the termination of the incubation. In all other respects the assay tube and its blank were handled in an identical manner.

After the termination of the incubation, the tubes were centrifuged at 27,000 *g*. The supernatant was chromatographed on silicic acid fiberglass sheets (Chromar 1000; Mallinckrodt Chemical Works, St. Louis, Mo.) in an ascending system composed of 0.03 M NH_4Cl, acetone, isopropanol, and *n*-butanol (2:2:2:3) for 75 min. In this system, cyclic AMP migrates close to the solvent front, ATP remains close to the origin, and AMP and ADP migrate at intermediate rates. The cyclic AMP area was eluted in 0.7 ml water, and 0.2 ml each of 0.25 M $Ba(OH)_2$ and 0.25 M $ZnSO_4$ was added. After centrifugation at 27,000 *g*, the supernatant was transferred to scintillation vials. To these was added 15 ml of scintillation fluid prepared by adding 2400 ml toluene and 1250 ml Triton X-100 to 100 ml Permafluor 25X (Packard Instrument Co., Inc., Downers Grove, Ill.). Specimens were counted 10 times for 10 min each time with about 27% efficiency for ^{3}H and 80% for ^{32}P in a Packard 3320 Tri-Carb liquid scintillation spectrometer. Only a 0.7% spillover of ^{32}P counts into the ^{3}H channel was observed, and there was no spillover of ^{3}H counts into the ^{32}P channel. The amount of cyclic AMP formed was corrected for losses on the basis of recovery of cyclic AMP-^{3}H. The amount of cyclic AMP found in each blank was subtracted from the corresponding incubate. Adenyl cyclase activity is expressed as picomoles cyclic AMP formed per milligram protein per 20 minutes. The identity and purity of the isolated cyclic AMP were established by methods previously described by Dorrington and Baggett (6).

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (7). Statistical analysis employed the Student *t* test.

The ACTH preparation, containing an activity of 57 USP U/mg, was a gift of Parke, Davis & Co. (Detroit, Mich.). Thyroid-stimulating hormone (TSH) (NIH-S6 and NIH-B5), luteinizing hormone (LH) (NIH-S16 and NIH-B7), and follicle-stimulating hormone (FSH) (NIH-S8 and NIH-P1) were kindly provided by Dr. A. Wilhelmi and Dr. L. Reichert of Emory University. TSH prepared by Armour Laboratories, Inc. (Glendale, Calif.) was also used in some experiments. Epinephrine, norepinephrine, and glucagon were from Sigma Chemical Co. (St. Louis, Mo.). Vasopressin was from Parke, Davis & Co., pork insulin from Eli Lilly & Co. (Indianapolis, Ind.) and asparaginyl^{1}-valyl^{5}-angiotensin II from Ciba Corp. (Summit, N. J.). Growth hormone (Raben type) was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). Parathyroid hormone and thyrocalcitonin were gifts of Dr. P. L. Munson. α-ATP-^{32}P was purchased from International Chemical & Nuclear Corporation (Burbank, Calif.) and cyclic AMP-^{3}H from Schwartz Bio Research Inc. (Orangeberg, N. J.).

RESULTS

Distribution of adenyl cyclase activity in normal adrenals and in the adrenocortical carcinoma. Enzyme activity was present in each of the particulate fractions obtained by centrifugation, both in normal adrenals (Table I) and in the tumor (Table II). Activity was lowest in particles sedimenting at 105,000 *g*. Although a small amount of activity was detected in initial experiments employing 105,000 *g* supernatant fractions, this observation could not be reproducibly documented.

Responses of adrenal cancer and normal adrenal adenyl cyclase to ACTH and NaF. Each of the particulate fractions of the tumor and of the adrenals was stimulated by ACTH (Tables I and II). The responses of the 105,000 *g* particles were small. Dose-response curves employing 1000 *g* particles from the tumor and from normal adrenals are shown in Fig. 1. Responses of the tumor to any given concentration of ACTH were generally greater than responses of normal adrenals, an observation noted for each tissue fraction. The lowest stimulatory concentration of ACTH for both tissues was 0.051 U/ml.

NaF also stimulated adenyl cyclase activity in each particulate fraction of the tumor and of normal adrenals (Tables I and II). However, the effects of NaF on the tumor were small, whereas NaF produced eightfold or more stimulation of the normal adrenal enzyme. This rather marked difference in the responses of the tumor and of the normal adrenal enzyme was noted for NaF concentrations ranging from 10 to 40 mmoles/liter.

TABLE I
Adenyl Cyclase Activity in Adrenal Homogenates and Centrifuged Fractions

Tissue fraction	Cyclic AMP formed		
	Basal	ACTH‡	NaF§
	pmoles/mg protein per 20 min (Mean ±SEM)		
Whole Homogenates	463 ±88 (17)*	1218 ±95 (10)	3721 ±460 (7)
1000 *g* particles	300 ±29 (14)	1213 ±148 (7)	5649 ±718 (6)
10,000 *g* particles	256 ±29 (11)	990 ±251 (6)	3962 ±464 (6)
105,000 *g* particles	39 ±5 (12)	100 ±21 (6)	2194 ±320 (6)

* Number of experiments. Each of the n (17) pooled results is the mean of an assay done in duplicate.
‡ ACTH, 0.25 U/ml.
§ 40 mM NaF.

Responses of adrenal cancer adenyl cyclase to catecholamines. To further characterize the properties of the tumor cyclase, a wide variety of other hormones were tested for effects on enzyme activity. Unexpectedly, the tumor cyclase was stimulated by epinephrine and by norepinephrine. Results of experiments employing 1000 *g* particles are summarized in Table III, and dose-response curves for each of the hormones are shown in Fig. 2. The lowest effective concentration for each of the hormones was 10^{-6} moles/liter. Normal adrenal adenyl cyclase was unresponsive to the catecholamines at concentrations as high as 10^{-4} moles/liter.

Response of adrenal cancer adenyl cyclase to TSH. The tumor cyclase was also stimulated by each preparation of TSH tested. Results of experiments with 1000 *g* particles are summarized in Table III, and a dose-response curve is shown in Fig. 3. TSH was active at concentrations as low as 0.0036 USP U/ml. The possibility that these responses were due to contamination of TSH preparations by ACTH was excluded by the observation that even 1000-fold increases in the concentration of the TSH preparations (3.6 U/ml) failed to have any effect on normal adrenal adenyl cyclase activity.

Tests for additive effects of different hormones in stimulating adrenal cancer adenyl cyclase. Possible additive effects of ACTH, TSH, and epinephrine on tumor cyclase activity were looked for, employing high concentrations of each of the hormones. Table IV shows results of two such experiments with 1000 *g* tumor particles. No additive effects were observed.

Other hormones tested. Growth hormone, angiotensin II, vasopressin, insulin, parathyroid hormone, thyrocalcitonin, and glucagon failed to produce statistically significant stimulation of the adenyl cyclase of the adenocortical cancer or of normal adrenals (Table V). FSH preparations (NIH-S8 and NIH-P1) and LH preparations (NIH-S16 and NIH-B7) significantly stimulated the tumor cyclase (Tables II and V). The lowest effective concentration of FSH, 0.23 NIH/ml, contained about 0.01 USP U of contaminating TSH/ml. The lowest active LH concentration, 0.35 NIH U/ml, contained 0.003 USP U/ml of TSH activity. It is, therefore, uncertain whether the activity of the LH and FSH preparations was due to intrinsic effects of these hormones on the adrenal cancer cyclase or to TSH contained in these preparations.

TABLE II

Adenyl Cyclase Activity in Tumor Homogenates and Centrifuged Fractions

Tissue fraction	Cyclic AMP formed		
	Basal	ACTH‡	NaF§
	pmoles/mg protein per 20 min (*Mean* ±SEM)		
Whole Homogenate	457 ±46 (17)*	1558 ±223 (10)	596 ±122 (7)
1000 *g* particles	513 ±57 (15)	3144 ±514 (9)	781 ±202 (7)
10,000 *g* particles	281 ±52 (12)	1387 ±492 (7)	461 ±97 (7)
105,000 *g* particles	26 ±6 (12)	70 ±17 (7)	107 ±5 (7)

* Number of experiments. Each of the n (7) pooled results is the mean of an assay done in duplicate.
‡ ACTH, 0.2 U/ml.
§ 40 mM NaF.

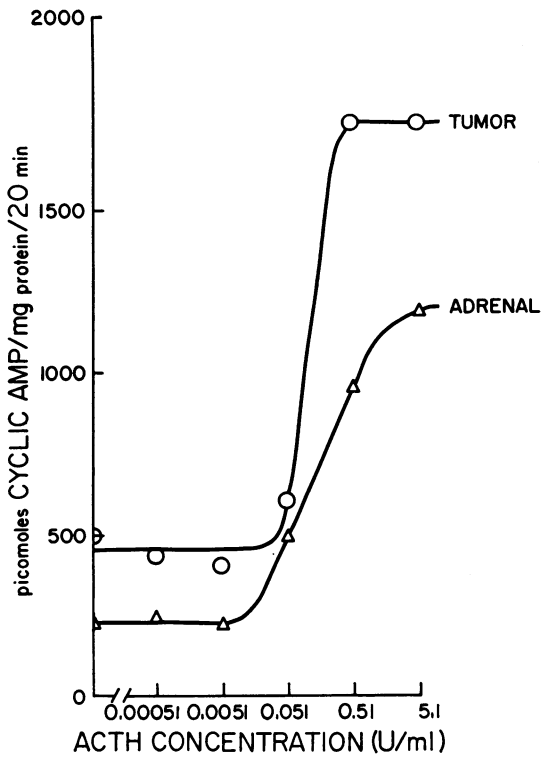

FIGURE 1 Response of adenyl cyclase activity of 1000 *g* particles from the adrenal cancer and from normal adrenals to varying concentrations of ACTH. The ACTH concentrations are shown on a log scale on the horizontal axis. Responses are on the vertical axis.

DISCUSSION

The characteristics of the adenyl cyclase of a transplantable corticosterone-producing adrenocortical cancer and of normal adrenals of the rat have been compared. Enzyme activity was distributed throughout particulate fractions of each tissue sedimenting at centrifugal forces up to 105,000 *g*. Activity in each particulate fraction of tumor and adrenals was stimulated by ACTH. The lowest effective concentration of ACTH in each tissue was 0.05 USP U/ml, or if this is estimated on a molar

TABLE III

Adenyl Cyclase Responses of 1000 g Particles of Adrenal Cancer to Various Hormones

Hormone	Cyclic AMP formed	*P* value*
	pmoles/mg protein per 20 min (*Mean* ±SEM)	
Basal	364 ±49 (11)‡	
ACTH, 5.1 USP U/ml	1301 ±234 (9)	<0.001
TSH, 3.5 USP U/ml	880 ±184 (6)	<0.01
FSH, 2.3 NIH U/ml	683 ±158 (7)	<0.02
LH, 3.5 NIH U/ml	502 ±65 (5)	<0.01
Epinephrine, 10^{-4} moles/liter	1411 ±246 (7)	<0.001
Norepinephrine, 10^{-4} moles/liter	1873 ±81 (2)	<0.001

* *P* value is for comparison of hormone response with basal cyclase activity.

‡ Number of experiments is shown in parentheses. Observations in each experiment were done in duplicate.

basis, approximately 10^{-6} moles/liter. The responses of the tumor to any given concentration of ACTH were somewhat greater than responses of normal adrenals. This difference might be accounted for by the heterogeneous composition of the adrenals, since no attempt to remove adrenal medulla was made. Tumor and adrenal particles were also stimulated by NaF. The normal adrenals demonstrated marked eightfold or greater increases in activity as NaF concentrations of 10–40 moles/liter, while the tumor demonstrated only small responses. Once again this difference could be due to the heterogeneous composition of the adrenals with medullary components largely accounting for the NaF responses. Alternatively the very low NaF responses of the tumor could reflect an abnormality of its adenyl cyclase system.

The most striking abnormality of the tumor adenyl cyclase was its unexpected stimulation by catecholamines and by TSH. Epinephrine and norepinephrine stimulated the tumor enzyme at concentrations as low as 10^{-6}moles/liter, comparable with the minimally effective concentrations of ACTH in this system. TSH stimulated adenyl cyclase activity in adrenal tumor preparations at levels as low as 0.0036 U/ml. It can be estimated that this represents a TSH concentration of approximately 10^{-6}moles/liter. The likelihood that the activity of any of these preparations was due to ACTH contamination was excluded by their failure to affect normal adrenal adenyl cyclase activity at concentrations 100- to 1000-fold greater than those required to stimulate the tumor enzyme. The tumor enzyme was also stimulated by preparations of LH and FSH. However, these preparations contained TSH contamination at levels sufficient to have accounted for their stimulation of the tumor cyclase. Further studies with more highly purified preparations of LH and FSH will be needed to determine whether one or both of these hormones do in fact have any effect on the tumor enzyme. Tumor adenyl cyclase was unresponsive to a wide variety of other hormones (Table V). ACTH was the only hormone preparation that stimulated normal adrenal adenyl cyclase. The results showing responses of tumor adenyl cyclase to TSH, epinephrine, and norepinephrine as well as to ACTH, suggest either that the receptor portion of the tumor adenyl cyclase has undergone structural alteration with consequent loss of hormonal specificity or that the tumor possesses additional "ectopic" adenyl cyclase receptors. The presence of ectopic adenyl cyclase receptors might be analogous to the synthesis of ectopic hormones by certain tumors (8). In this case, however, the ectopic substances would be incorporated in the structure of the cell rather than being delivered into the circulation.

The physiological significance of the responses of the tumor cyclase to hormones other than ACTH remains

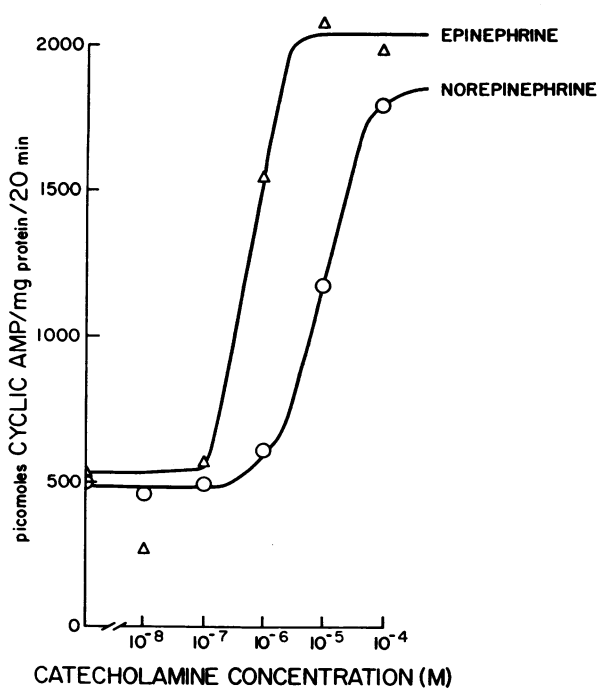

FIGURE 2 Response of adenyl cyclase activity of 1000 *g* particles from the adrenal cancer to varying concentrations of epinephrine and norepinephrine.

to be ascertained. Concentrations of cyclic AMP in the tumor in vivo are about 4 nmoles/g, whereas concentrations in the nontumorous adrenals of the tumor-bearing animals are about 2 nmoles/g (5). The administration of large doses of ACTH results in about a 50-fold increase in cyclic AMP levels in nontumorous adrenals, but tumor levels rise only to about 6 nmoles/g. Although the capacity of the tumor to increase cyclic AMP levels in vivo is limited compared with that of normal adrenals, concentrations of cyclic AMP in the range 4–6 nmoles/g in normal adrenals would be associated with a high rate of steroidogenesis (2), whereas the tumor is comparatively inefficient as a steroidogenic tissue even at these cyclic AMP levels (5). It would appear, therefore, that the limited steroidogenic responses of the adrenal cancer to ACTH are due principally to defects in the systems acted upon by cyclic AMP rather than to limitations in cyclic AMP formation. This conclusion is supported by the observation that the addition of large concentrations of exogenous cyclic AMP in vitro fail to have any substantial effect on tumor steroidogenesis while markedly stimulating normal adrenal steroidogenesis (5). It is possible that the tumor is already responding at its maximal capacity to its endogenous cyclic AMP levels. The factors maintaining these endogenous cyclic AMP levels are presently not clear, but the results of the present experiments raise the possibility that a variety of hormones other than ACTH may be influencing tumor cyclic AMP concentrations. Therefore, removal of these other hormones (by hypophysectomy) or inhibition of their activity (by adrenergic-blocking drugs) might reduce tumor cyclic AMP levels and rates of steroidogenesis. Such experiments remain to be performed.

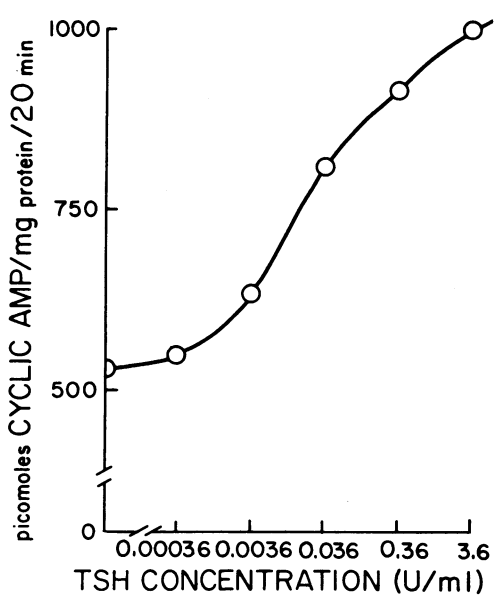

FIGURE 3 Response of adenyl cyclase activity of 1000 *g* particles from the adrenal cancer to varying concentrations of TSH.

TABLE IV

Adenyl Cyclase Responses of 1000 g Particles of Adrenal Cancer to ACTH, TSH, and Epinephrine and to Their Combinations

Hormone	Cyclic AMP formed	
	Experiment 1	Experiment 2
	pmoles/mg protein per 20 min	
Basal	692	220
ATCH, 5.1 USP U/ml	2254	536
TSH, 3.6 USP U/ml	1593	444
Epinephrine, 10^{-4} moles/liter	2230	707
ACTH + TSH	2226	452
TSH + epinephrine	2094	519
ACTH + epinephrine	2538	538
ACTH + TSH + epinephrine	2287	457

It is interesting to speculate that the apparently autonomous behavior of some endocrine tumors might be due to hormonal influences other than the usual ones for the tissue of origin. Thus, for example, although glucocorticoid-producing adrenal neoplasms suppress ACTH secretion, other hormones might maintain tumor cyclic AMP levels and rates of steroidogenesis. Whether or not exogenous ACTH administration stimulated tumor steroidogenesis to a higher level would depend on whether or not the tumor's steroidogenic mechanisms were already responding maximally to tissue cy-

TABLE V

Response of 1000 g Particles of Normal Adrenals and of Adrenal Cancer to Various Hormones

Hormone	Normal adrenal	Adrenal tumor
	% of basal value	
Basal	100	100
Epinephrine, 10^{-4} moles/liter	89	388*
Norepinephrine, 10^{-4} moles/liter	91	514*
TSH, 3.6 USP U/ml	92	214*
FSH, 2.3 NIH U/ml	92	188*
LH, 3.5 NIH U/ml	91	138*
Glucagon, 0.35 mg/ml	72	101
Parathyroid hormone, 771 USP U/ml	69	84
Thyrocalcitonin, 17.6 MRC U/ml	88	103
Vasopressin, 50 pressor U/ml	105	117
Insulin, 0.6 mg/ml	75	102
Angiotensin II, 0.013 mg/ml	98	114
Growth hormone, 2 mg/ml	106	97

* Significantly greater than basal values at P value of less than 0.05.

clic AMP levels. In ACTH-unresponsive neoplasms (most adrenocortical carcinomas), steroidogenesis may already be responding at its maximal capacity to cyclic nucleotide levels, and therefore even if ACTH adminis tration were to further elevate nucleotide levels, no increase in steroidogenesis could occur. On the other hand, in ACTH-responsive tumors (some adenomas), tumor cyclic AMP levels might be maintained by other hormones at levels sufficient to sustain steroidogenesis even at elevated levels but not at the tumors maximal capacity. The administration of ACTH would further elevate cyclic nucleotide levels with consequent increases in steroidogenesis.

It should be noted that in another adrenocortical neoplasma, a mouse tumor maintained in tissue culture, responses of the tumor cyclase to hormones other than ACTH were not observed (9). Therefore the aberrant adenyl cyclase responeses of the tumor we have studied may not be a general phenomenon. On the other hand, there is evidence that abnormal adenyl cyclase responses of endocrine tumors are not unique to the adrenocortical cancer utilized in the present studies. For example, some pheochromocytomas increase catecholamine production in response to glucagon. Recent studies have revealed that these pheochromocytomas possess a glucagon-sensitive adenyl cyclase system, whereas normal adrenal medulla cyclase is unresponsive to this hormone (10). In addition, we have studied several human endocrine tumors to date and have found unexpected hormonal responses of their adenyl cyclase systems. For example, the adenyl cyclase of a parathyroid adenoma was stimulated by glucagon.[2] Further studies will be needed to clarify the physiological significance of abnormal cyclase responses of endocrine tumors and to determine if any diagnostic or therapeutic use can be made of this information.

[2] Schorr, I., and R. L. Ney. Unpublished observations.

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