ACTH and Prostaglandin Receptors in Human Adrenocortical Tumors

APPARENT MODIFICATION OF A SPECIFIC COMPONENT OF THE ACTH-BINDING SITE

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Abstract The failure of certain adrenal tumors to respond to ACTH was investigated in vivo by administration of corticotropin-(1-24)-tetracosapeptide ($ACTH_{1-24}$) and dexamethasone and in vitro by studying the binding properties of $ACTH_{1-24}$ and prostaglandin E_1 (PGE_1) and their effect on adenylate cyclase activity of the tumors' crude membranes; in addition, in five cases the stimulation of cortisol production in isolated adrenal cells by both hormones and dibutyryl cyclic adenosine 3',5'-monophosphate (cAMP) was also studied. The results obtained in 13 hormone-producing tumors of the human adrenal cortex, i.e. 10 carcinomas and 3 adenomas, were compared with those found in normal human adrenal glands.

According to the adenylate cyclase responses to $ACTH_{1-24}$ and PGE_1, the tumors fall into different categories. In the first group are six tumors in which the adenylate cyclase was stimulated by both $ACTH_{1-24}$ and PGE_1; in addition specific binding could be demonstrated for the two hormones in all six. The binding affinity for ^{125}I-$ACTH_{1-24}$ was found to be about 10 times higher than that for ^{125}I-$ACTH_{11-24}$. In the one tumor in which the experiment was performed, bound ^{125}I-$ACTH_{1-24}$ was displaced by $ACTH_{1-10}$. These results are similar to the ones found in normal human adrenal preparations. For two tumors of the group in which ACTH did not increase steroidogenesis in vivo, the biochemical abnormality might be located beyond cAMP formation.

A second group encompasses six tumors in which the steroidogenesis in vivo and the adenylate cyclase activity were insensitive to $ACTH_{1-24}$ but in which the enzyme was stimulated by PGE_1 and NaF. However, these preparations bound ^{125}I-$ACTH_{1-24}$ and ^{125}I-$ACTH_{11-24}$, the binding affinity being similar for both peptides but 10 times lower than the one found in normal adrenal cortex for ^{125}I-$ACTH_{1-24}$. In the only case of this group where it was tested, $ACTH_{1-10}$ did not displace bound ^{125}I-$ACTH_{1-24}$. This result strongly suggests the possibility of a modification or a loss of the receptor site that binds the N-terminal sequence (1-10) of ACTH, the biologically active part of the molecule.

In the last tumor, both PGE_1 and ACTH were unable to stimulate adenylate cyclase activity and steroid production in a preparation of isolated adrenal cells, although steroidogenesis was stimulated by dibutyryl cAMP. No specific binding for PGE_1 could be demonstrated. However, ^{125}I-$ACTH_{1-24}$ and ^{125}I-$ACTH_{11-24}$ were found to be bound to the tumor with the same affinity.

INTRODUCTION

It is well known that in most of the human adrenocortical tumors ACTH does not stimulate steroidogenesis (1). The biochemical anomaly responsible for this failure of stimulation of steroidogenesis by ACTH is still unknown. The same ACTH insensitivity has also been shown in vivo and in vitro for several types of adrenocortical tumors of the rat. In the tumor first described by Snell and Stewart (2), the main biochemical abnormality seemed to be located beyond the formation of cyclic AMP (3) since neither ACTH nor cyclic AMP could increase steroidogenesis (4–6), although adenylate

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TABLE I

Urinary Excretion of Steroids in Patients with Adrenocortical Tumors

Patients	Sex	Age	Etiology	Tumor wt	Basal		Dexamethasone*		ACTH‡	
					17-KS	17-OCHS	17-KS	17-OCHS	17-KS	17-OCHS
		yr		*g*	*mg/24 h*		*mg/24 h*		*mg/24 h*	
1	F	$1\frac{1}{12}$	R. carcinoma§	165	24–28	1.1–1.7	26	1.3	—	—
2	M	6	L. carcinoma (recurrence)	70	18	1.4	20	1.4	—	—
3	F	7	R. carcinoma	320	50	25				
4	F	18	L. carcinoma	170	400–600	9–12	399	0.5	540	5
5	F	36	R. carcinoma	210	157	8	185	30	180	—
6	F	38	L. carcinoma	1,350	94–159	47–91	84	45	114	59
7	F	39	L. carcinoma	750	200	43	134	19	210	47
8	F	47	L. carcinoma	400	216–270	24–28	206	24	215	30
9	F	51	R. carcinoma	510	39	4.8	31	2.5	25	3.9
10	M	54	R. carcinoma	1,815	150	19	127	—	202	33
11	F	7	L. adenoma	72	39–72	10–16	41	14	50	15
12	F	25	R. adenoma	80	65–75	5–10	69	3	72	22
13	M	53	L. adenoma	120	5–8	15–20	6	21	10	60

* After dexamethasone, 8 mg/day for 3 days.

‡ After Synacthen-Depot®, 1 mg/day for 2 days.

§ R., right; L., left.

cyclase activity in subcellular fractions was stimulated by ACTH (3). In other mutant adrenal cell lines studied by Shimmer (7, 8) the anomaly was localized in the cell membrane since adenylate cyclase in adrenal subcellular preparations was not stimulated by ACTH, but cyclic AMP was still able to stimulate steroidogenesis.

The purpose of this work was to establish whether in human adrenocortical tumors abnormalities of the ACTH receptors on the plasma membrane could be demonstrated. Therefore, we have studied the interaction of ACTH and also of prostaglandin E_1 (PGE_1)[1] with subcellular fractions prepared from 13 tumors, and the results have been compared to those obtained with the same type of preparations from normal human adrenals.

METHODS

Materials. 13 human adrenocortical tumors were used for this study (10 carcinomas and 3 adenomas). The type and weight of the tumors as well as the output of 17-ketosteroids (17-KS) and 17-hydroxycorticosteroids (17-OHCS), measured by the method described by Few (9) and Glenn and Nelson (10), respectively, are shown in Table I. In all the patients, there were signs of virilization except in patient 10, who presented signs of marked feminization, and in patient 13, who did not present any clinical signs of excessive hormone secretion. Normal adrenal glands (confirmed histologically) were obtained from women during surgery for breast cancer. None of them had been given any previous antimitotic, hormonal, or X-ray therapy. A pool of 10 normal adrenal glands was used for all the binding and adenylate cyclase studies. The adrenal tumors were obtained at surgery.

Corticotropin-(1-24)-tetracosapeptide ($ACTH_{1-24}$), corticotropin-(11-24)-tetradecapeptide ($ACTH_{11-24}$), and corticotropin-(1-10)-decapeptide ($ACTH_{1-10}$) were generously provided by Drs. Rittel and Desaulles (Ciba-Geigy AG., Basel, Switzerland). Labeling of both ACTH's with ^{125}I was performed by the method described previously (11). PGE_1 was a gift from Dr. J. E. Pike, Upjohn Company, Kalamazoo, Mich. [^{3}H]PGE_1 (sp act 68.5 Ci/mol) was purchased from New England Nuclear, Boston, Mass. Microfine silica, Quso G-32, was obtained from Philadelphia Quartz Co., Philadelphia, Pa. Other chemicals were of reagent grade.

Membrane preparation. Before homogenization, the medulla of normal adrenals and the necrotic portions of the tumor were removed. Normal adrenal or tumor tissue was homogenized in 20 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose with a Potter-Elvejehm glass homogenizer (10 strokes) followed by filtration through two layers of gauze and 5 strokes of the loose pestle in a Dounce homogenizer. The homogenate was centrifuged twice at 800 *g* for 10 min. The sediment was collected, and the supernate was centrifuged at 20,000 *g* for 30 min. The pellet obtained at 20,000 *g* was washed with 20 mM Tris-HCl buffer, pH 7.4, centrifuged, and kept in small aliquots (crude membranes). In certain tumors, highly purified preparations of plasma membranes were made (and confirmed by electron microscopy) by the method of Finn, Widnell, and Hofmann (12). This method was inapplicable to normal human adrenals be-

[1] *Abbreviations and trivial names used in this paper:* $ACTH_{1-10}$, corticotropin-(1-10)-decapeptide; $ACTH_{1-24}$, corticotropin-(1-24)-tetracosapeptide; $ACTH_{11-24}$, corticotropin-(11-24)-tetradecapeptide; androstenedione, androst-4-ene-3, 17-dione; cortisol, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; DcAMP, dibutyryl cyclic adenosine 3',5'-monophosphate; dehydroepiandrosterone (DHA), 3β-hydroxyandrost-5-en-17-one; DHAS, DHA sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; 17-KS, 17-ketosteroids; 17-OHCS, 17-hydroxycorticosteroids; PGE_1, PGE_2, prostaglandins E_1 and E_2, respectively.

TABLE II

Plasma Concentrations of Several Steroids in Patients with Adrenocortical Tumors

Patients	Basal				Dexamethasone*				ACTH‡			
	DHAS	DHA	Androstenedione	Cortisol	DHAS	DHA	Androstenedione	Cortisol	DHAS	DHA	Ahdrostenedione	Cortisol
	μg/100 ml				*μg/100 ml*				*μg/100 ml*			
3	—	0.3	—	22		0.05		16				
6	1,520	2.9	—	29	1,340	2.8	6.1	31	1,500	2.5	6	26
7	1,551	3.4	5.9	16					1,384	3.2	4.7	10
10	960	1.9	0.9	28					910	1.3	0.8	22

* After dexamethansone, 8 mg/day for 3 days.

‡ After Synacthen-Depot ®, 1 mg/day for 2 days.

cause of too low a yield (less than 0.1 mg membrane protein per gram of adrenal tissue). All the preparations were kept in liquid nitrogen and thawed shortly before use. In these conditions the binding capacity and the adenylate cyclase activity remained unaltered at least for 4 mo.

Binding studies. Measurement of the binding for [^{3}H]-PGE_1, ^{125}I-$ACTH_{1-24}$, and ^{125}I-$ACTH_{11-24}$ to normal adrenal particulate fractions has been described in detail elsewhere (13, 14). Briefly, 0.25 ml of the membrane protein preparation in 20 mM Tris-HCl buffer pH 7.4 containing 1% albumin and the labeled hormone ([^{3}H]PGE_1 or ^{125}I-ACTH) was incubated at 4°C. When equilibrium was reached (30 min for ^{125}I-ACTH and 90 min for [^{3}H]PGE_1), the sample was layered over 1 ml of 20 mM Tris-HCl buffer, pH 7.4, 0.25 M sucrose containing 2% albumin and centrifuged immediately at 50,000 *g* for 10 min at 0°C. The supernate was removed, and the pellet was retained. The pellet containing [^{3}H]PGE_1 was dissolved in 0.1 ml of Soluene® (Packard Instrument Co., Inc., Downers Grove, Ill.) for 1 h at 60°C and transferred with 0.4 ml of methanol in 10 ml Bray's scintillation fluid. The pellet containing ^{125}I-ACTH was counted in a well-type scintillation counter.

All binding determinations were performed in six replicates, three containing only the radioactive hormone and three containing the radioactive plus the unlabeled hormones (10 μg of PGE_1 or 200 μg of $ACTH_{1-24}$). The mean of the three latter figures, which is an estimate of nonspecific binding, was subtracted in each instance from the average of the first three. In all the experiments the nonspecific binding represented 1–6% of the total binding for ^{125}I-$ACTH_{1-24}$ and ^{125}I-$ACTH_{11-24}$ and 8–12% of the total binding of [^{3}H]PGE_1.

Assay for ACTH degradation. The degradation of ^{125}I-$ACTH_{1-24}$ was measured according to a method described previously (11). Briefly, after incubation of ^{125}I-$ACTH_{1-24}$ with the crude membrane preparation for 30 min at 4°C, the sample was layered over 2.5 ml of 20 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 2% albumin and centrifuged at 50,000 *g* for 20 min at 0°C. Only the 1st ml of the supernate was aspirated and kept (unbound fraction). The pellet was washed once with 2 ml of the same buffer and centrifuged at 50,000 *g* for 20 min. The supernate was discarded and the pellet was resuspended in 0.5–1.0 ml of 1% acetic acid, shaken for 30 min at 22°C, and centrifuged at 50,000 *g* for 10 min. The supernate (bound fraction) was removed and neutralized with 1 N NaOH. About 70–80% of the radioactivity present in the pellet was recovered in the acetic acid fraction. The degradation of unbound and bound ACTH was measured by absorption of the labeled material on microfine silica (Quso G-32) and from its ability to bind to fresh adrenal crude membranes. The results were expressed as the percentage of the hormone that remains intact in relation to a control specimen that was incubated under the same conditions but without membranes.

Preparation of isolated cells. Isolated adrenal cells from normal adrenals and tumors were prepared according to a modification (15) of the method described by Sayers, Swallow, and Giordano (16). An aliquot of the cell suspension stained with trypan blue showed more than 85% of viable cells. Steroid production by the isolated adrenal cells was measured after 2 h at 37°C in 0.8 ml of Krebs-Ringer bicarbonate containing 0.2% glucose and 4% albumin. ACTH or other substances were added in a volume of 0.1 ml of vehicle (0.9% sodium chloride, pH 3, for ACTH and dibutyryl cyclic AMP [DcAMP] and 0.9% sodium chloride containing 0.1% ethanol for PGE_1). The mixture was gassed with 95% oxygen and 5% CO_2 and incubated in a Dubnoff metabolic shaker.

Assay of steroids. Cortisol was measured by a competitive protein-binding method (17). Testosterone, androstenedione, and dehydroepiandrosterone (DHA) after separation and purification on Celite columns (Johns-Manville Products Corporation, New York) were measured by a sensitive radioimmunoassay (18). The first steps of purification of DHA sulfate (DHAS) were performed as described (19). After solvolysis, the compound was measured by radioimmunoassay as DHA.

Enzymatic activities. 5′-Nucleotidase was determined according to Heppel and Hilmor (20), adenosine 3′,5′-monophosphate phosphodiesterase was assayed by the method of Rutten, Schoot, and de Pont (21), and adenylate cyclase was measured as described elsewhere (13). Protein content was estimated by the method of Lowry, Rosebrough, Farr, and Randall (22) with bovine serum albumin as standard.

RESULTS

In vivo effects of ACTH and dexamethasone administration. The urinary excretion of steroids after administration of ACTH or dexamethasone is shown in Table I and the plasma content of steroids under the same conditions in patients where the test was available is shown in Table II. In addition, in patients 11 and 13 a perfusion of Synacthen® (Ciba Pharmaceutical Company, Summit, N. J.), 250 μg in 500 ml of saline, was

TABLE III

Cortisol Production by Isolated Cells from Human Adrenocortical Tumors

	Subjects				
	3	6	7	9	10
	ng/2 h per 10^6 cells				
Basal	26±3*	21±1.2	22±3	3.6±0.6	7.5±1.2
ACTH, 30 nM	247±15‡	19±2	24±3	2.9±0.4	9.8±2.1
ACTH, 3 μM	258±18‡	21±2	23±4	2.6±0.3	8.9±2.3
PGE_1, 60 nM	200±15‡	65±14‡	63±8‡		
PGE_2, 2 μM		63±8‡		9.6±1.3‡	8.6±1.8
DcAMP, 1 mM	286±28‡	128±14‡	181±12‡	37.4±2.4‡	26.6±3.1‡

* Mean±SD (three observations).

‡ $P < 0.05$ compared to the first value in its own column.

administered for 6 h. Plasma cortisol was measured at 0, 2, 4, and 6 h. The following results were obtained–patient 11: 27, 20, 25, and 20 μg/100 ml, respectively; patient 13: 17, 51, 84, and 65 μg/100 ml, respectively. These results suggest that patients 4–8, 10, and 11 did not respond to either of the two tests. Patient 13 responded to ACTH but not to dexamethasone. The results of patient 12 are difficult to explain.[2]

Cortisol production by isolated adrenal cells (Table III). Since the interpretation of the ACTH stimulation test in vivo was difficult, we decided, in the last five patients of our study (nos. 3, 6, 7, 9, and 10), to investigate the response of isolated adrenal cells to ACTH, PGE_1, and DcAMP. Stimulation of cortisol production with ACTH was obtained in only one case (no. 3).

[2] The interpretation of the tests using ACTH and dexamethasone administration in patients presenting an adrenal tumor is difficult for two reasons: (*a*) The output of urinary steroids may vary from one day to another in the same patient within limits as high as 100%. (*b*) Some adrenal tumors of the virilizing type present a low secretion of cortisol, probably not submitted to the feed-back control mechanism which regulates the function of the normal adrenal. This could explain the responses to the tests in the patient 12, who, besides the adenoma, had an adrenal of normal macroscopic and microscopic aspect.

TABLE IV

Adenyl Cyclase Activity of Crude Membranes (20,000 g) of Human Adrenocortical Tumors under Several Conditions

	Cyclic AMP*					
Patients	Basal	ACTH (10 μM)	PGE_1 (10 μM)	ACTH (10 μM) + PGE_1 (10 μM)	EGTA (0.1 mM)	NaF (6 mM)
	pmol/10 min per mg protein					
1	258±40	380±25‡	348±51‡	425±20§	530±52‡	3,820±140‡
2	103±15	185±21‡	145±16‡	212±23§	147±14‡	1,581±102‡
3	122±16	360±32‡	302±38‡	460±37	181±15‡	3,580±180‡
4	236±32	520±48‡	850±68‡	1,110±90§	395±42‡	9,300±244‡
5	659±50	793±50‡	1,029±70‡	1,120±74§	—	6,580±180‡
6	198±21	204±28	309±32‡	304±28	274±22‡	2,950±130‡
7	288±15	277±28	373±22‡	384±23	360±18‡	4,852±210‡
8	91±8	88±9	138±11‡	135±11	188±15‡	2,789±160‡
9	463±40	349±29	1,120±80‡	635±50	617±52‡	8,540±280‡
10	135±13	141±15	140±12	137±9	—	3,980±160‡
11	135±13	132±14	202±19‡	—	—	2,548±140‡
12	220±28	222±25	294±30‡	296±42	292±38‡	1,475±70‡
13	882±86	2,586±145‡	1,496±155‡	3,172±134§	1,202±103‡	7,089±310‡
Normal human adrenal	358±20	848±40‡	910±36‡	1,271±80§	690±50‡	3,720±142‡

* Mean±SD (12 observations).

‡ $P<0.05$ compared to the first value in its own line.

§ $P<0.05$ compared to the values of ACTH or PGE_1.

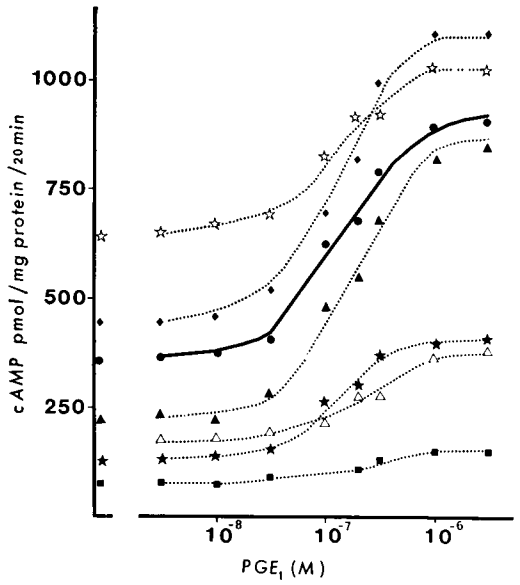

FIGURE 1 Effects of increasing concentrations of PGE_1 on adenylate cyclase activity of crude membranes obtained from normal human adrenals (●) and tumors 3 (★), 4 (▲), 5 (☆), 6 (△), 8 (■), and 9 (♦).

This preparation also responded to DcAMP and PGE_1. Namely, the stimulation obtained with 3×10^{-8} M ACTH was similar to that induced by 10^{-3} M DcAMP and higher than that due to 2×10^{-6} M PGE_1. In the other four cases ACTH was unable to stimulate the cortisol production, but a stimulatory effect could be obtained by using DcAMP and PGE_1 in three of the tumors (nos. 6, 7, and 9), while in tumor 10 only DcAMP increased slightly the cortisol production.

Adenylate cyclase activity. The enzymatic activity of crude membranes under basal conditions varied from one tumor to another (Table IV). With the exceptions of tumors 5, 9, and 13, it was generally lower than the activity found in the same preparation from normal human adrenals. Otherwise, the adenylate cyclase of the tumor preparations had similar characteristics to that from the normal adrenals. In the presence of a fixed concentration of ATP (0.53 mM), increasing amounts of Mg^{2+} stimulated the enzyme and reached a plateau towards 4 mM. On the other hand, in the presence of a fixed concentration of Mg^{2+} (7.5 mM) the maximal enzymatic activity was obtained with 0.8–1 mM ATP. Further increase of the ATP concentration inhibited the adenylate cyclase activity. This inhibition was virtually complete at 3 mM ATP. Ca^{2+} inhibited the enzyme and the inhibition was maximal at 10 mM.

Ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) and NaF stimulate the adenylate cyclase activity in crude membrane preparations of adrenal gland from several species (23). In the normal human adrenal cortex the stimulation was maximal with concentrations of 0.1 mM EGTA and 6 mM NaF. At these concentrations, the stimulation was about 80 and 1,000% of the basal activity, respectively. In the tumor preparations the percent stimulation induced by EGTA was similar to that observed in normal adrenals, but the one induced by NaF was generally higher (Table IV).

PGE_1 stimulates the adenylate cyclase activity in particulate preparations of human adrenals (13). Maximal stimulation (100% of basal activity) is obtained with 5×10^{-8} M of the hormone. All tumors except no. 10 were sensitive to PGE_1 (Table IV and Fig. 1). However, the percentage of stimulation varied from one case to another. The stimulation was normal in cases 3–5, 9, and 13, low but significant in cases 1, 2, 6–8, 11, and 12, and undetectable in case 10.

In crude membranes of normal human adrenals ACTH is able to induce a 100% stimulation of basal adenylate cyclase activity when used at a 10^{-5} M concentration; higher values lead to a reversal of the stimulation (Fig. 2). Half-maximal stimulation is obtained with 6×10^{-7} M of ACTH.

In the tumors of our present investigation (Table IV) the stimulation of adenylate cyclase activity induced by 10^{-5} M ACTH was unobtainable for seven cases (nos. 6–12), low but significant in three cases (nos. 1, 2, and 5) and normal in the last three cases (nos. 3, 4, and 13). The half-maximal stimulation was obtained with about 6×10^{-7} M ACTH. This value is calculated from experiments performed in tumors 3–5, where dose-response curves to increasing ACTH concentrations could be established (Fig. 2).

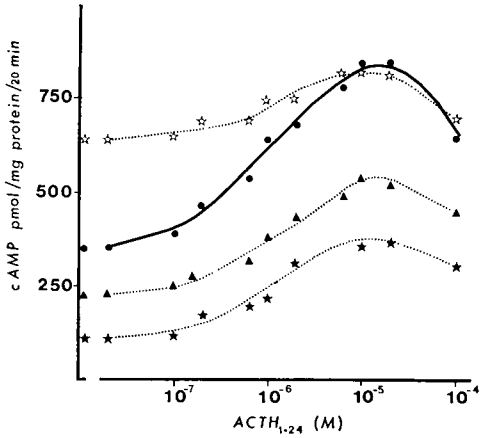

FIGURE 2 Effect of increasing concentrations of $ACTH_{1-24}$ on adenylate cyclase activity of crude membranes obtained from normal human adrenals (●) and tumors 3 (★), 4 (▲), and 5 (☆).

TABLE V

Phosphodiesterase Activity in Crude Membranes of Human Adrenocortical Tumors

Patients	Cyclic AMP degraded
	nmol/15 min per mg protein
1	14
2	26
3	38
5	28
6	34
7	42
8	44
9	17
10	21
12	30
13	33
Normal human adrenal	87

Crude membranes (100 μg of protein) were incubated in 0.2 ml of 66 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mM CyclicAMP at 37°C for 15 min. The values are mean of three replications.

An additive effect of ACTH and PGE_1 on adenylate cyclase stimulation has been shown for normal ovine and human adrenals (Table IV and reference 3). The same additive effect can be described for the tumors responsive to both hormones (nos. 1–5 and 13). Concentrations (10^{-5} M) of ACTH and PGE_1 responsible for maximal stimulation of adenylate cyclase activity when added separately induce a further stimulatory effect when they are added simultaneously. In the tumors insensitive to ACTH, the PGE_1 stimulation is not changed by the presence of ACTH, except in tumor 9, in which ACTH inhibits in part PGE_1 stimulation.

Other hormones have been shown to be effective in stimulating the adenylate cyclase activity of the particulate fraction from the tumor 494 described by Snell and Stewart (2). The tumors tested in the present study (nos. 2 and 8–10) did not respond to concentrations as high as 10^{-5} M of epinephrine, luteinizing hormone, glucagon, and insulin (data not shown).

The inability of $ACTH_{1-24}$ to stimulate the accumulation of cyclicAMP in tumors 6–12 could be attributed to one or several of the following abnormalities:

(*a*) Increase of the phosphodiesterase activity of the tumor crude membranes. This hypothesis is very unlikely since the stimulation of adenylate cyclase induced by other stimuli was normal or higher than normal and, when measured, the phosphodiesterase activity was lower than in normal adrenals (Table V). These results are similar to those reported by Sharma (24), who showed a lower phosphodiesterase activity in adrenal cortical tumors than in the normal adrenal in rats.

(*b*) Abnormal distribution of adenylate cyclase activity in subcellular fractions of adrenal tumors. The distributions of 5′-nucleotidase and adenylate cyclase in the different subcellular fractions of tumors (nos. 5 and 12) are given in Table VI and compared to results obtained in normal human adrenals. These two enzymatic activities were present in all of the particulate fractions in both tumors and the normal adrenal. The distribution of adenylate cyclase activity differed from the one observed in the normal adrenal (Table VI). However, this table clearly shows that in none of the subcellular fractions obtained from tumor 12 was the adenylate cyclase sensitive to ACTH.

TABLE VI

Distribution of 5′-Nucleotidase and Adenylate Cyclase Activities in the Subcellular Fractions of Normal and Tumor Human Adrenals

	Normal adrenal				Tumor 12				Tumor 5			
		Adenylate cyclase*				Adenylate cyclase*			Adenylate cyclase*			
	5′-Nucleotidase	Basal	ACTH (10 μM)	NaF (6 mM)	5′-Nucleotidase	Basal	ACTH (10 μM)	NaF (6 mM)	Basal	ACTH (10 μM)	NaF (6 mM)	
	mol P_i liberated/h per mg protein	*pmol/20 min per mg protein*			*mol P_i liberated/h per mg protein*	*pmol/20 min per mg protein*			*pmol/20 min per mg protein*			
Homogenate	1.2	109	359	1,980	2.8	244	250	2,260	271	241	1,645	
800 *g*	2.2	92	370	2,480	4.2	590	610	7,651	303	363	2,277	
20,000 *g*	4	330	829	3,690	1.7	215	218	1,520	640	784	6,580	
105,000 *g*	4.8	103	117	1,400	4.1	589	579	5,921	353	357	1,785	
Purified membranes‡	—	—	—	—	15.3	1,608	1,641	20,280	—	—	—	

* Mean value of three replications.

‡ Prepared by the method of Finn et al. (12).

TABLE VII

Degradation of ^{125}I-$ACTH_{1-24}$ by Crude Membranes of Human Adrenocortical Tumor

Patients	Percentage of hormone remaining intact			
	Unbound		Bound	
	Adsorption to Quso	Binding to fresh membranes	Adsorption to Quso	Binding to fresh membranes
1	11	3	97	107
2	73	54	99	114
3	19	9	96	107
4	22	14	98	110
5	14	5	95	104
6	36	24	98	111
7	28	17	98	109
8	52	33	99	114
9	20	8	97	107
10	47	33	99	110
11	39	28	97	108
12	18	6	97	106
13	17	10	96	104
Normal human adrenal	45±2.8*	31±3	99±6	110±5

Crude membranes (1.4 mg of protein/ml) were incubated 30 min at 4°C with ^{125}I-$ACTH_{1-24}$ (22 nM). Bound and unbound ^{125}I-$ACTH_{1-24}$ were separated by centrifugation and bound ^{125}I-$ACTH_{1-24}$ extracted by 1% acetic acid (see Methods). Degradation of ^{125}I-$ACTH_{1-24}$ in both fractions was measured by absorption to Quso and ability to bind to fresh adrenal membranes as described under Methods.

* Mean value of six replications.

(*c*) Increase degradation of $ACTH_{1-24}$ by tumor crude membranes. It has been recently demonstrated that crude membrane preparations of normal adrenals of several species can degrade ACTH (11). This degradation is independent from the binding, and it only affects the ACTH that is not bound to its receptor. Preparations of crude membranes prepared from adrenal tumors also degraded unbound ^{125}I-$ACTH_{1-24}$ but did not degrade bound ^{125}I-$ACTH_{1-24}$ (Table VII). The degradation of unbound ACTH estimated by binding to fresh membranes was, in all the tumors (as observed in normal adrenals), greater than that estimated by absorption to Quso.[3] The degree of degradation of ACTH varied from one tumor to another, but these quantitative variations could not explain why in certain tumors adenylate cyclase was not stimulated by ACTH. The degradation system was less potent in tumors 6, 8, 10, and 11 although in all of them ACTH did not stimulate adenylate cyclase. In the other tumors (except for no. 2) the degradation was greater than normal even in cases where adenylate cyclase responded to ACTH.

(*d*) Modification of the ACTH binding receptor in tumor crude membranes. Specific binding of ^{125}I-$ACTH_{1-24}$ and ^{125}I-$ACTH_{11-24}$ to crude membranes of tumoral origin was shown by the same criteria as those used for normal adrenal preparations (14): (i) Bound radioactivity was displaced only by $ACTH_{1-24}$ and some of its analogues (see below). Insulin, glucagon, and PGE_1 at high concentrations (10^{-5} M) were without effects. (ii) Heating the membranes at 60°C for 30 min or pretreatment with trypsin (250 μg/mg of membrane protein) completely inhibited the binding.

According to these criteria all the membranes prepared from the tumors specifically bound both labeled ACTH's. However, the apparent binding affinity of $ACTH_{1-24}$ for the tumors in which the adenylate cyclase was not stimulated by ACTH was about 10 times lower than that for normal adrenals and for tumors in which the adenylate cyclase was stimulated by ACTH. On the other hand, the binding affinity of $ACTH_{11-24}$ was similar for both normal adrenals and all the tumors and about 10 times lower than the one of $ACTH_{1-24}$ in a

[3] In two cases, normal adrenals and tumor 10, the degradation of bound and unbound ^{125}I-$ACTH_{1-24}$ was also measured by the ability of bound and unbound hormone to stimulate the cortisol secretion by the isolated ovine adrenal cells. The percentage of hormone remaining intact in the unbound fraction was 28 and 30 in normal adrenal and tumor 10, respectively, and in the bound fraction the percentage was 112 and 110, respectively. These results are similar to those found when the degradation was measured by the ability to bind to fresh adrenal membranes (Table VII).

TABLE VIII

Inhibition of Binding of ^{125}I-$ACTH_{1-24}$ by $ACTH_{1-24}$ (First Column) and of ^{125}I-$ACTH_{11-24}$ by $ACTH_{11-24}$ (Second Column)

Subjects	^{125}I-$ACTH_{1-24}$	^{125}I-$ACTH_{11-24}$
	μM	
1	0.34	
2	0.55	
3	0.41	2.5
4	0.42	3.8
5	0.62	
6	2.1	4.1
7	3.9	2.5
8	3.9	
9	4.1	5.4
10	6.2	7.1
11	4	5.2
12	2.7	
13	0.24	
Normal human adrenal	0.32	4.1

The table gives the concentrations of $ACTH_{1-24}$ and $ACTH_{11-24}$ required to displace 50% of the bound labeled hormone.

normal gland (Table VIII). Figs. 3 and 4 are representative experiments of the displacement of ^{125}I-$ACTH_{1-24}$ and ^{125}I-$ACTH_{11-24}$ by $ACTH_{1-24}$ and $ACTH_{11-24}$, respectively, in normal adrenals, one tumor that responds to ACTH (no. 3), and four ACTH nonresponding tumors (nos. 6–9).

In normal adrenal preparations $ACTH_{1-10}$ displaced bound ^{125}I-$ACTH_{1-24}$ but not bound ^{125}I-$ACTH_{11-24}$ (14). Owing to insufficient quantities of unlabeled $ACTH_{1-10}$, we could study the effect of this peptide in only two cases. The results in Table IX show that $ACTH_{1-10}$ displaced bound ^{125}I-$ACTH_{1-24}$ in tumor 3 (ACTH responder) but did not in no. 6 (ACTH nonresponder) and suggest that the binding of the sequence 1–10 of ACTH may be specifically impaired in tumors 6–12.

TABLE IX

Inhibition of the Binding of ^{125}I-$ACTH_{1-24}$ to Crude Membranes by $ACTH_{1-10}$

Subjects	Control	+$ACTH_{1-10}$(0.1 mM)
3	100±3*	71±4‡
6	100±4*	116±6‡
Normal human adrenal	100±3*	65±3‡

Membranes were incubated 30 min at 4°C in 0.25 ml of 20 mM Tris-HCl (pH 7.4) containing 1% albumin and 1 nM ^{125}I-$ACTH_{1-24}$ with or without $ACTH_{1-10}$ (0.1 mM). Results for specific binding obtained under control conditions are given an arbitrary value of 100.

* Mean±SD (six observations).

‡ Mean±SD (three observations).

TABLE X

Specific Binding of [^3H]PGE_1 (12 nM) to Crude Membranes Obtained from Adrenocortical Tumors

Subjects	[^3H]PGE_1 bound
	*pmol/mg protein**
1	0.18±0.05
2	0.16±0.04
3	0.85±0.10
4	1.6 ±0.12
5	0.94±0.09
6	0.64±0.07
7	0.49±0.06
8	0.34±0.02
9	1.10±0.11
10	0‡
11	0.52±0.03
12	0.45±0.07
13	1.10±0.12
Normal human adrenal	1.20±0.09

Membranes (about 100 μg of protein) were incubated at 4°C for 90 min in 0.25 ml of 20 mM Tris-HCl, pH 7.4, containing 1% albumin and 12 nM [^3H]PGE_1. Specific binding of the hormone was measured as described under Methods.

* Mean±SD (nine observations).

‡ See text.

However, these studies do not exclude an associated abnormality of the coupling system in this group of tumors.

Specific binding of [^3H]PGE_1 to crude membranes obtained from adrenal tumors. Since the adenylate cyclase of tumor 10 was not stimulated by PGE_1 (Table IV), we decided to investigate whether any abnormality of the binding receptor of the hormone could be demonstrated in this tumor. As in normal human adrenal preparations (13) a specific binding of [^3H]PGE_1 was observed in all the tumors, except in no. 10 (see below). The specificity of the binding was shown by the fact that in the presence of 10 μg of PGE_1, a 88–92% decrease in total radioactivity bound was noted. ACTH, insulin, and glucagon at high concentrations (10^{-5} M) had no effect (data not shown). On the contrary, 200 μg of crude membranes from tumor 10 in the presence of 1.2×10^{-8} M [^3H]PGE_1 bound only 1.1±0.3% of the total radioactivity, and after addition of 10 μg of unlabeled PGE_1 the radioactivity bound was the same (1±0.4%). In the other tumors the specific binding varied from one tumor to another and in general was much lower than that of normal human adrenals (Table X).

DISCUSSION

In the normal human adrenal ACTH is essential for the continuous production of glucocorticoids and an-

drogens (25). The first detectable step in the mechanism of action of ACTH is the binding of this hormone to specific adrenocortical receptors (26, 27). This interaction is followed by a stimulation of the adenylate cyclase enzyme which is responsible for an increase in intracellular cyclic AMP level (28). This cyclic nucleotide, by mechanisms still not understood, increases steroidogenesis (29). The failure of certain adrenal tumors to respond to ACTH in vivo despite the existence of a high rate of steroidogenesis may suggest either the presence of one or several abnormalities in the biochemical sequence initiated by the hormone or that steroidogenesis in these tumors is already accelerated to a maximal rate by endogenous ACTH or other factors.

The study in vitro of human adrenal tumors has shown a great heterogeneity in all the parameters that have been studied. However, according to the adenylate cyclase response to ACTH and PGE_1 the tumors investigated here can be divided into three categories: (*a*) tumors in which the adenylate cyclase was stimulated by both ACTH and PGE_1 (nos. 1–5 and 13); (*b*) those in which adenylate cyclase was stimulated by PGE_1 but not by ACTH (nos. 6–9, 11 and 12); (*c*) the one in which adenylate cyclase was stimulated neither by PGE_1 nor by ACTH (no. 10).

In the first group of tumors adenylate cyclase was stimulated by the same factors as in the normal adrenal. The quantitative variations observed might be accounted for by the heterogenous composition of the crude membrane preparations used in both normal adrenals and adrenal tumors and/or by an abnormality of the adenylate cyclase system. In this group, only in two cases (nos. 4 and 5) was an insensitivity to ACTH demonstrated in vivo. The results in these two patients were similar to those observed in the 494 adrenocortical tumor of the rat where ACTH was unable to stimulate steroidogenesis in vivo and in vitro (3–6) whereas adenylate cyclase in particulate preparations was stimulated by the hormone (3). Since in this tumor cyclic AMP did not stimulate steroidogenesis (4, 5), it has been suggested (3) that the main anomaly responsible for the insensitivity to ACTH would occur after the formation of cyclic AMP. As our results suggest but do not confirm (since we could not test the effect of DcAMP on steroid production), the same anomaly could be present in some human adrenal cortical tumors. However, the similarity between human and rat tumors is far from complete. We have not found receptors for epinephrine and luteinizing hormone in the human tumors as were found in the rat tumor (30). It also remains to be established if the multiple anomalies in the mechanism of action of ACTH on the rat tumor, described by Sharma (6, 31, 32), are present in this group of human tumors.

The failure of ACTH to stimulate the cyclic AMP accumulation in our second group of tumors cannot be explained by an increased activity of the nucleotide phosphodiesterase (Table V). Moreover, it is probably not due to an anomaly of the catalytic sites of adenylate cyclase, since the enzymatic activity was stimulated by PGE_1, EGTA, and NaF. Therefore, the nonresponse to ACTH is probably related to a membrane anomaly. Adenylate cyclase insensitivities to specific hormones have already been reported for ACTH by Shimmer (8) in a mutant adrenal cell line and for thyroid-stimulating hormone by Macchia, Meldolesi, and Shiariello (33) in a rat thyroid tumor. In these cases, as in human adrenal tumors, the adenylate cyclase was stimulated by NaF.

The membrane anomaly in our second group of tumors could be located in the hormone binding receptor and/or at the level of the system coupling discriminator and catalytic sites. Although the binding of $^{125}I\text{-}ACTH_{1\text{-}24}$ is specific in those tumors, the first hypothesis cannot be eliminated.

Recent studies concerning the relationship between the structure and the function of ACTH (12, 27, 34–37) have shown that the peptide sequences necessary for the binding and for the biological action of this hormone are localized in two distinct parts of the molecule. The C-terminal ($ACTH_{11\text{-}24}$) is important for binding (12) but has no biological action (37) whereas the N-terminal sequence ($ACTH_{1\text{-}10}$) has a very low binding affinity but is essential for biological action. In addition, it has been shown (11) that in normal adrenals the binding affinity of $^{125}I\text{-}ACTH_{1\text{-}24}$ is about 10 times higher than that of $^{125}I\text{-}ACTH_{11\text{-}24}$ and that $ACTH_{1\text{-}10}$ displaces bound $^{125}I\text{-}ACTH_{1\text{-}24}$ but not $ACTH_{11\text{-}24}$. This could be explained by the existence in the normal adrenal membranes of two distinct binding sites for the ACTH molecule, one related to the 1-10 sequence and the other one to the 11-24 sequence.

Data (Figs. 3 and 4 and Tables VIII and IX) from the binding studies in tumors of our second group (where adenylate cyclase does not respond to ACTH) clearly show that the binding affinity for $ACTH_{1\text{-}24}$ is much lower than normal. It is similar to that for $ACTH_{11\text{-}24}$ and not 10 times higher as in the normal gland or in the tumors sensitive to ACTH. This loss could not possibly be related to a modification of the site which binds the 11-24 sequence of the hormone since the binding affinity of the $ACTH_{11\text{-}24}$ remained normal. Alternatively, the failure of $ACTH_{1\text{-}10}$ to displace bound $^{125}I\text{-}ACTH_{1\text{-}24}$ in the tumor 6 strongly suggested that, at least in this example, the defect is probably due to a modification or a loss of the membrane component which normally binds the 1-10 sequence of ACTH. However, the possibility of an associated anomaly of the coupling system of the ACTH receptor cannot be ruled out by our study; nevertheless, this

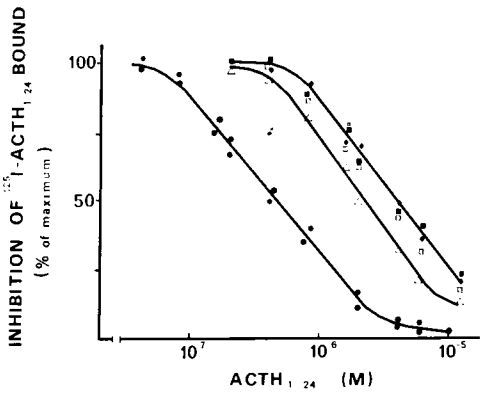

FIGURE 3 Displacement of bound ^{125}I-ACTH$_{1-24}$ by ACTH$_{1-24}$. Membranes were incubated 30 min at 4°C in 0.25 ml of 20 mM Tris-HCl containing 1% albumin and 1 × 10^{-9} M ^{125}I-ACTH$_{1-24}$ and the indicated concentrations of unlabeled ACTH$_{1-24}$. Crude membranes were obtained from normal human adrenals (●) and tumors 3 (*), 6 (△), 7 (□), 8 (■), and 9 (♦).

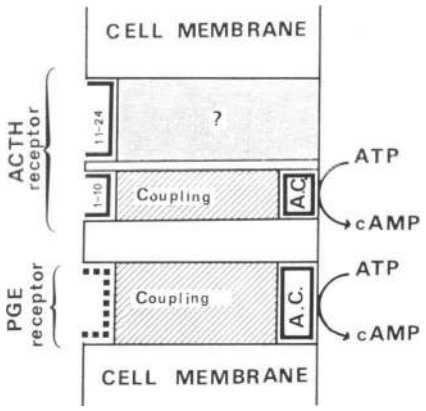

FIGURE 5 Proposed model for ACTH and PGE$_1$ receptors in adrenal membranes. ACTH receptor is composed of two binding sites. One binds the 11-24 sequence, the other one the 1-10 sequence. The binding site and the coupling system for PGE$_1$ are independent of those for ACTH. A. C., adenylate cyclase.

anomaly would not be extended to the coupling system of the PGE$_1$ receptor since the hormone stimulated the adenylate cyclase activity of those tumors.

All the results presented so far for both tumors and normal adrenals can be reconciled in a diagram representing the receptors for ACTH and PGE$_1$ such as the one proposed in Fig. 5. Although no biological action has yet been described for ACTH$_{11-24}$, a possible role for the 11-24 sequence could be to increase the binding affinity of ACTH and therefore to allow the sequence 1-10 to fit in the second site. Only when this second site is occupied would the adenylate cyclase be stimulated.

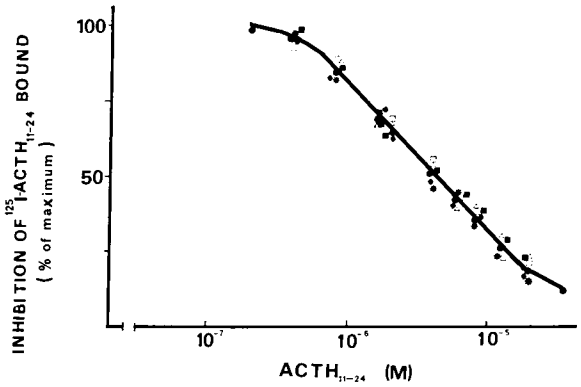

FIGURE 4 Displacement of bound ^{125}I-ACTH$_{11-24}$ by ACTH$_{11-24}$. Membranes were incubated 30 min at 4°C in 0.25 ml of 20 mM Tris-HCl containing 1% albumin and 1.2 × 10^{-9} M ^{125}I-ACTH$_{11-24}$ and the indicated concentrations of unlabeled ACTH$_{11-24}$. Crude membranes were obtained from normal human adrenals (●) and tumors 3 (*), 6 (△), 7 (□), 8 (■), and 9 (♦).

This model would also explain the case of the tumor (no. 10) of the last category which, in addition to the anomalies of the ACTH receptor, as for the second group, presents also an associated defect of the PGE$_1$ receptor. The PGE$_1$ did not stimulate the adenylate cyclase of this preparation, and no significant binding could be observed with the tritiated hormone; however, it could not be demonstrated whether the defect consisted in a modification or a loss of the PGE$_1$-binding site, which could or could not be associated with an anomaly of its coupling system.

It can be concluded from our study that in human adrenocortical tumors, a loss of sensitivity to ACTH can be related to a variety of biochemical abnormalities. They can affect different components of the ACTH receptor-adenylate cyclase complex or the message initiated by the formation of cyclicAMP. Further work in progress in this laboratory indicates that their detailed observation and analysis, despite an apparent heterogeneity, will give valuable information concerning the physiological role of the different peptide sequences of ACTH in the normal adrenal cell.

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