

# Direct Effects of Thyrotropin-releasing Hormone, Cyproheptadine, and Dopamine on Adrenocorticotropin Secretion from Human Corticotroph Adenoma Cells In Vitro

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**ABSTRACT** In an attempt to delineate the mechanism and the site of action of cyproheptadine and dopaminergic agonists as well as hormones including thyrotropin-releasing hormone (TRH) and hydrocortisone, the effects of these substances on ACTH secretion from corticotroph adenoma cells in culture were examined. Dispersed cells of pituitary adenomas obtained at surgery from four patients with Nelson's syndrome and one subject with Cushing's disease formed a monolayer and actively secreted ACTH into the medium. When TRH (0.1  $\mu$ M) was added to the medium, a significant increase in ACTH secretion was demonstrated by adenoma cells from two patients who responded to TRH preoperatively. Moreover, a dose-response relationship between TRH concentrations and ACTH secretion was observed. Incubation of cells with cyproheptadine (1 or 0.1  $\mu$ M) resulted in a significant decrease in ACTH release, and inhibited stimulation produced by TRH in one experiment. This effect of cyproheptadine was blocked when equimolar concentrations of serotonin was coincubated, whereas serotonin by itself did not affect ACTH secretion. Dopamine (0.1  $\mu$ M) lowered ACTH accumulation in the medium, which was blocked by the addition of haloperidol. When hydrocortisone was added to the culture, dose-dependent suppression of ACTH secretion was demonstrated. TRH at an equimolar concentration reversed this effect, but, failed to overcome the inhibition induced by a higher concentration of hydrocortisone in cells from one adenoma studied. Cultured normal corticotrophs obtained from a patient

with metastatic breast cancer, on the other hand, did not show any response to these substances, except for hydrocortisone. We suggest that TRH, cyproheptadine, and dopamine affect ACTH secretion in patients with ACTH-producing pituitary adenomas by their direct action on the adenoma.

## INTRODUCTION

Long-term administration of cyproheptadine, a serotonin antagonist, was found by Krieger et al. (1) to suppress the circulating levels of ACTH and to offer concomitant beneficial clinical effects in patients with Cushing's disease. Subsequent studies confirmed the efficacy of this drug in the management of some, though not all, patients with Cushing's disease and Nelson's syndrome (2-8). Bromocriptine, a dopaminergic agonist, likewise has been shown to lower plasma ACTH levels in some patients, and its clinical use is currently being evaluated (9-15). These works opened the possibility of medical management of the disorder. The mechanism by which these drugs inhibit ACTH secretion, however, remains unknown.

It has been recently shown, on the other hand, that pituitary microadenomas are found in the majority of patients with Cushing's disease and that selective removal of the tumor corrects hypercortisolism in these patients (16-19). Although drugs such as cyproheptadine and bromocriptine that modulate the activity of neurotransmitters may act on the central nervous system, a direct action on a pituitary adenoma has not been ruled out. We have recently demonstrated, in an in vitro perfusion system, that thyrotropin-

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<sup>1</sup>Abbreviation used in this paper: TRH, thyrotropin-releasing hormone. The nomenclature proposed by Li (20) is used to refer the various fragments of ACTH.

releasing hormone (TRH)<sup>1</sup> and bromocriptine act directly on pituitary adenomas obtained from patients with acromegaly to affect growth hormone and prolactin release (21). By analogy, we hypothesized that cyproheptadine and bromocriptine may possess a direct action on ACTH-producing pituitary adenoma cells of Cushing's disease and Nelson's syndrome. To determine whether this hypothesis is correct, we examined the effect of these drugs on ACTH secretion, using cultured adenoma cells obtained from patients with Nelson's syndrome and Cushing's disease, and compared their responses with the responses of normal human corticotrophs. Furthermore, the effect of TRH and hydrocortisone, which have been shown to modulate plasma ACTH levels in these patients, were tested in this system.

## METHODS

**Subjects.** Four patients with Nelson's syndrome (patients 1-4) and one subject with Cushing's disease (patient 5) were studied. These five patients consisted of a man and four women, aged 20-47 yr. The patients with Nelson's syndrome developed marked hyperpigmentation 4-8 yr after bilateral adrenalectomy, in all four, abnormalities in sella turcica were demonstrated by polytomography. In the remaining patient, the diagnosis of Cushing's disease was established by clinical findings and confirmed by combined adrenal function tests. None of the subjects received any medication that could affect ACTH secretion, except for glucocorticoid at a replacement dose, which was given to subjects with Nelson's syndrome. All in vivo studies were performed in the morning after overnight fast,  $\geq 20$  h after the last dose of the usual replacement therapy in patients with Nelson's syndrome. The patients were kept recumbent for at least 1 h before and throughout the study except for standing to void. Heparinized blood was collected through an indwelling catheter placed in an antecubital vein.

**Test procedures.** After taking a control sample, 500  $\mu$ g of synthetic TRH (Tanabe Pharmaceutical Co., Osaka, Japan) was injected intravenously as a single bolus. Blood samples were then collected at 15, 30, 60, 90, and 120 min after the injection. After two base-line samples were taken (-30 and 0 min), 4 mg of cyproheptadine (Nippon Merck-Banyu Co., Tokyo, Japan) was administered orally or 50 mg of hydrocortisone hemisuccinate (Solu-cortef; Japan Upjohn Ltd., Tokyo, Japan) was injected intravenously as a single bolus. Blood samples were collected at 30-min intervals for the subsequent 4 h. Blood samples taken under exactly the same conditions without drug administration served as a control to examine the spontaneous fluctuation of plasma ACTH levels. Blood was transferred to chilled tubes and immediately centrifuged. Plasma was stored at  $-20^{\circ}\text{C}$  until assayed.

**Monolayer tissue culture of pituitary adenomas.** Pituitary adenomas were obtained at surgery from these patients. Light microscopy revealed that all of the pituitary adenomas were chromophobe. A normal anterior pituitary was obtained from a woman aged 49 yr at the time of hypophysectomy for palliation of metastatic breast cancer. The method for preparing the dispersed cells employed in this study is a modification of the procedure originally described by Lambert et al. (22). Pituitary adenoma or normal pituitary tissue was cut into small pieces and incubated with 10 ml of trypsin-collagenase solution with gentle magnetic stirring at  $37^{\circ}\text{C}$ . This solution contained 0.25 g trypsin

(Difco Laboratories, Detroit, Mich.), 20 mg collagenase (type IV; Worthington Biochemical Corp., Freehold, N. J.) and 0.05 g glucose in 100 ml of  $\text{Ca}^{++}$ -free,  $\text{Mg}^{++}$ -free phosphate buffered saline (pH 7.6). After 10 min of incubation, residual tissues were allowed to settle. The supernatant suspension containing dissociated cells were removed, diluted with cold culture medium, and kept on ice until the final centrifugation. Another 10 ml of trypsin-collagenase solution was added to the remaining tissue fragments and the digestion procedure was repeated two to three times until cells were completely dispersed. The supernatant fluids were then pooled and centrifuged at 150 g for 10 min. After decantation of the supernate, the cell pellet was washed with culture medium and again centrifuged. Cells were resuspended in an appropriate volume (usually 60 ml) of culture medium by drawing repeatedly through a Pasteur pipette. 2-ml aliquots containing  $\sim 100,000$  cells was planted in each plastic petri dish (35  $\times$  10 mm; Falcon Labware, Div. Becton-Dickinson & Co., Oxnard, Calif.), and incubated at  $37^{\circ}\text{C}$  in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . The culture medium consisted of Eagle's Minimum Essential Medium in Earle's solution (Grand Island Biochemical Co., Grand Island, N. Y.) containing 10% fetal calf serum, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin sulfate.

**Incubation of cells with test substances.** Incubation studies were started 48-72 h after planting, when the cells were well attached to dishes, and performed at 2-3-d intervals. Individual cultures were randomly allocated for each experiment. At least four cultures were used for the control and variables, and run simultaneously.

On the day of each experiment, the medium was replaced by 2 ml of Eagle's Minimum Essential Medium in Earle's solution containing 0.5% human albumin instead of fetal calf serum. Cells were incubated for 1 h at  $37^{\circ}\text{C}$  in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . The medium was then removed and cells were further incubated for 2 h in 2 ml of fresh medium with or without the following substances: TRH, hydrocortisone hemisuccinate, dopamine hydrochloride (Kyowa Hakkō Kogyo Co., Tokyo, Japan), haloperidol (Dai-Nippon Pharmaceutical Co., Osaka, Japan), cyproheptadine, serotonin creatinine sulfate (Tokyo-Kasei Kogyo Co., Tokyo, Japan), 5-hydroxytryptophan (Sigma Chemical Co., St. Louis, Mo.), and synthetic lysine vasopressin (Grade IV, 75 U/mg; Sigma Chemical Co.). After incubation, the medium was centrifuged at 150 g for 10 min, and the supernate was immediately frozen on dry ice and stored at  $-20^{\circ}\text{C}$  until analyzed. ACTH concentrations in the medium of both preincubation and experimental incubation were determined by radioimmunoassay. Results are expressed as the percentage of hormone secreted in the experimental incubation, compared with that secreted during the preincubation for individual cultures. For comparison, the mean values obtained in the control study were designated as 100%.

**Radioimmunoassay for ACTH.** Radioimmunoassay for ACTH was performed according to the method of Berson and Yalow (23), with minor modifications. Immunological materials for the radioimmunoassay were kindly supplied by the National Institute of Arthritis, Metabolism, and Digestive Diseases and the National Pituitary Agency. Synthetic  $\alpha_{1-39}$  ACTH was labeled with  $^{125}\text{I}$  by a chloramine-T method (24) and purified with QUSO G32 (Philadelphia Quartz Co., Philadelphia, Pa.). Varying amounts of unlabeled ACTH or incubation medium were placed in each tube and an appropriate amount of 0.1 M phosphate buffer, pH 7.4, including 0.5% human serum albumin, 0.5% 2-mercaptoethanol and 500 U/ml aprotinin was added to yield a volume of 300  $\mu\text{l}$ . To this was then added 100  $\mu\text{l}$  of diluted

anti-ACTH serum and labeled ACTH (2,500 cpm), and incubation was performed at 4°C for 5 d. Antibody-bound and free hormone were separated by use of polyethylene glycol (25). The lower limit of the sensitivity was 2 pg/tube. The coefficients of variation averaged 7.3% for intra-assay error and 11.4% for interassay error. When plasma samples were assayed, 20–200  $\mu$ l of plasma was used with a larger volume of assay buffer to yield the final volume of 1 ml. To validate the ACTH assay with raw plasma, 20, 50, or 200  $\mu$ l of unextracted plasma (five or six samples in each group) with various ACTH concentrations ranging from 51 to 7,400 pg/ml was assayed and the results were compared with the values obtained by radioimmunoassay after adsorption onto glass microbeads by the method of Rees et al. (26). When the ACTH concentrations in unextracted plasma were plotted on the ordinate and the estimates by the extraction method on the abscissa, a linear regression line with a slope of 0.98 and an intercept of 11 pg ( $r = 0.999$ ,  $n = 16$ ) was obtained, which demonstrates that raw plasma does not interfere significantly in the present ACTH radioimmunoassay.

**Statistical analysis.** Values in figures and text are given as the mean  $\pm$  SEM, unless otherwise specified. The significance of differences was calculated using Student's *t* test and analysis of variance.

## RESULTS

**Plasma ACTH response to TRH, cyproheptadine, and hydrocortisone.** Since several investigators have reported that there exists a fairly large variation in plasma ACTH levels throughout the day in patients with Cushing's disease and in those with Nelson's syndrome, the extent of the spontaneous fluctuation of plasma ACTH was examined in our patients by taking blood samples at 30-min intervals for 4 h. Time-to-time variation was unexpectedly small; the mean  $\pm$  SD plasma ACTH concentrations were 5,948  $\pm$  455 (patient 1), 3,795  $\pm$  427 (patient 2), 4,919  $\pm$  387 (patient 3),

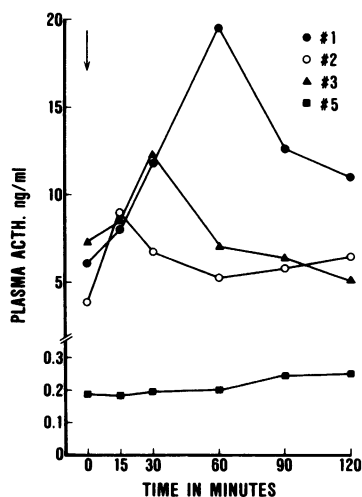


FIGURE 1 Plasma ACTH levels in patients with Nelson's syndrome (patients 1–3) and with Cushing's disease (patient 5) after an intravenous injection of TRH. TRH was administered at time zero at a dose of 0.5 mg as a single bolus.

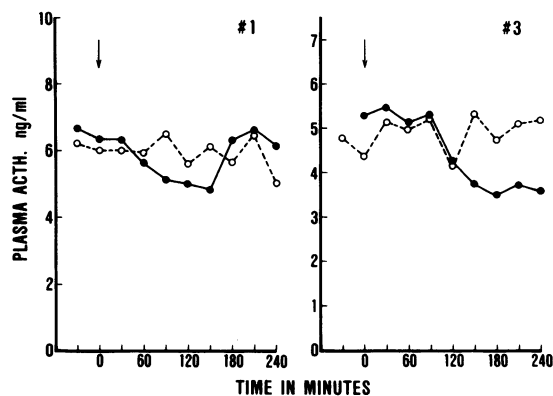


FIGURE 2 Plasma ACTH levels in two patients with Nelson's syndrome (patients 1 and 3) after cyproheptadine administration (●) in comparison with the levels on a control day (○). Cyproheptadine was administered at time zero at an oral dose of 4 mg.

and 225  $\pm$  25.1 pg/ml (patient 5), respectively. On the basis of this finding, the response was judged to be positive to a given stimulus in the following study when an elevation or a reduction in plasma ACTH concentrations exceeded twice the standard deviation from the mean value, that is, 15.1 (patient 1), 22.5 (patient 2), 15.7 (patient 3), and 22.3% (patient 5) of each control level, respectively.

Fig. 1 shows the changes in plasma ACTH concentrations in response to TRH injection in four patients. TRH-induced ACTH release in all of the three subjects with Nelson's syndrome tested (patients 1–3). Plasma ACTH peaked at 15–60 min after the injection and peak values were 319 (patient 1), 226 (patient 2), and 168% (patient 3) of their control levels, respectively. The patient with Cushing's disease (patient 5) showed a small gradual rise in plasma ACTH concentrations after TRH administration, but the pattern was different from other patients.

Fig. 2 shows the effect of cyproheptadine administration on plasma ACTH concentrations in two patients with Nelson's syndrome (patients 1 and 3). A single dose of cyproheptadine resulted in a slight but a significant decrease in plasma ACTH levels in both patients. The lowest values were obtained at 150 or 180 min after the administration, and corresponded to 73.6 (patient 1) and 67.3% (patient 3) of their control levels, respectively, which were lower than the mean-3 SD of spontaneous fluctuation in each patient. In one patient (No. 1), plasma ACTH levels tended to rise again at 180 min after the administration, whereas the suppressive effect of cyproheptadine was continued during the period of observation in the other (patient 3).

Fig. 3 depicts the changes in plasma ACTH levels after a single injection of hydrocortisone in three

patients with Nelson's syndrome. In all of the subjects, a significant decrease in plasma ACTH concentrations was observed at least 90 min after the administration. Plasma ACTH levels showed a progressive decline thereafter and the nadir, which was unequivocally observed at 240 min, reached 10.1 (patient 1), 33.1 (patient 2), and 14.5% (patient 3) of their control levels, respectively.

**ACTH secretion from cultured pituitary adenoma cells.** Dispersed cells of pituitary corticotroph adenomas formed a monolayer attaching to the culture dishes usually within the first 2 d. Fig. 4 shows the hourly secretion rates of ACTH on different culture days by corticotroph adenoma cells obtained from patient 4 with Nelson's syndrome. Synthesis and secretion of ACTH were well maintained throughout the period of observation, although a gradual decrease in ACTH release was seen when the culture was continued. The amounts of ACTH released into the medium in control incubations from all of the five cultured adenoma tissues studied are summarized in Table I. All the cultured cells continued, though declining, ACTH secretion over the entire period of experiment. The rates of ACTH release after incubation corresponded to  $69.2 \pm 5.7\%$  (mean  $\pm$  SEM,  $n = 12$ ) of the preincubation rates. The reason for this decrease is unexplained at present. It may be due to repeated cell washing and medium changing. Similar results have been observed by other investigators for ACTH and  $\beta$ -lipotropin secretion from cultured human corticotroph adenoma (27) and for  $\beta$ -lipotropin secretion from mouse pituitary tumor cells, AtT-20/D16v

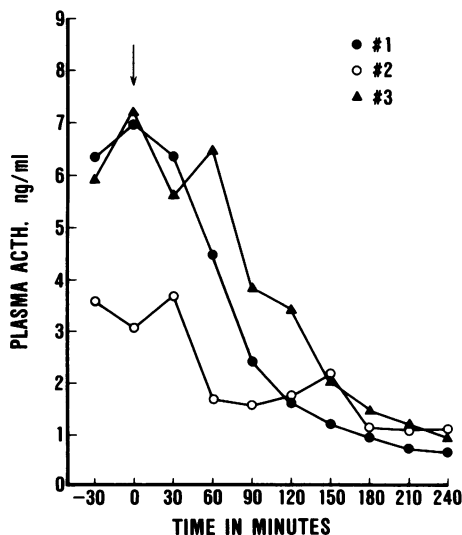


FIGURE 3 Plasma ACTH levels in three patients with Nelson's syndrome (patients 1-3) after an intravenous injection of hydrocortisone hemisuccinate. Hydrocortisone hemisuccinate was administered at time zero at a dose of 50 mg as a single bolus.

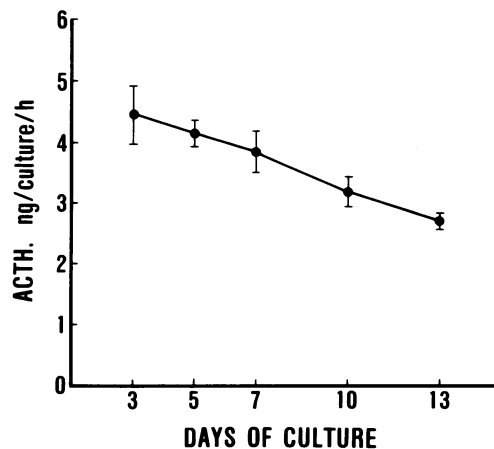


FIGURE 4 Hourly secretion rates of ACTH on different days in culture from corticotroph adenoma cells obtained from patient 4 who had Nelson's syndrome. Cells were incubated with fresh medium containing 0.5% human serum albumin for 1 h. Each group consists of four to eight dishes. Results are the mean  $\pm$  SEM.

(28). Nevertheless, our system appeared to be useful for the study of corticotroph adenomas at a cellular level, since the response of cells to a given stimulus was reproducible when examined on two different days in culture as described below (Fig. 6). When 20 ng of synthetic  $\alpha_h$  (1-39) ACTH was incubated at 37°C for 2 h with 2 ml of the medium containing 0.5% human albumin in the absence of cells, the mean recovery of radioimmunoassayable ACTH was  $96.9 \pm 1.9\%$  (mean  $\pm$  SEM,  $n = 6$ ).

**Effect of cyproheptadine on ACTH secretion from cultured pituitary adenoma cells.** The effect of cyproheptadine on ACTH release by cultured pituitary adenoma cells is illustrated in Fig. 5. When cyproheptadine was added to the incubation medium, ACTH secretion was significantly suppressed in all of the five experiments using different adenoma tissues. In three adenoma cells (Nos. 1-3) obtained from patients with Nelson's syndrome, cyproheptadine at a concentration of 0.1  $\mu$ M resulted in a significant decrease in ACTH released into the medium by 48.3 (No. 1) ( $P < 0.01$ ), 27.3 (No. 2) ( $P < 0.01$ ), and 12.6% (No. 3) ( $P < 0.05$ ), respectively, compared with each control. Although cyproheptadine at the same concentration was ineffective in lowering ACTH secretion in the remaining two adenoma tissues (Nos. 4 and 5), a significant decrease ( $P < 0.01$ ) in ACTH release was observed with a higher concentration (1  $\mu$ M). This inhibitory effect of cyproheptadine on ACTH secretion from cultured pituitary adenoma cells was blocked by the addition of serotonin at equimolar concentrations in two different adenoma cells (Nos. 3 and 5). Serotonin by itself (1 or 0.1  $\mu$ M), however, induced no significant effect on ACTH secretion. Similarly, 5-

**TABLE I**  
*Amounts of Immunoreactive ACTH Secreted into the Medium by Cultured Corticotroph Adenoma Cells in Control Incubations in Each Experiment*

Patient	Age	Sex	Medium ACTH concentrations					
			First experiment*		Second experiment		Third experiment	
			Preincubation	Experimental incubation	Preincubation	Experimental incubation	Preincubation	Experimental incubation
	yr		ng/ml					
1	20	M	50.3±4.3	47.9±4.4	40.9±4.0	52.0±3.6	18.4±1.4	26.4±2.3
2	47	F	14.9±2.2	26.9±4.5	—	—	—	—
3	34	F	7.75±0.79	16.3±1.9	8.42±0.60	11.8±0.76	6.90±1.0	13.6±3.0
4	30	F	2.08±0.11	2.99±0.39	1.92±0.17	2.31±0.53	1.35±0.07	1.54±0.13
5	46	F	1.09±0.05	1.03±0.04	0.73±0.02	0.69±0.05	—	—

Cells were incubated at 37°C in 2 ml of Minimum Essential Medium (Eagle) in Earle's solution containing 0.5% human serum albumin (preincubation). After 1 h, the medium was removed and cells were further incubated with 2 ml of fresh medium for 2 h (experimental incubation). Each group consisted of at least four cultures. The results are expressed as the mean±SEM.

\* First, second, and third experiments were conducted on days 3–4, 5–7, and 7–13 in culture, respectively.

hydroxytryptophan, a precursor of serotonin, failed to affect ACTH release from the cultured adenoma cells (Nos. 3 and 5).

*Effect of dopamine on ACTH secretion from cultured adenoma cells.* Fig. 6 shows the effect of dopamine on ACTH release by cultured pituitary adenoma cells. In all of the experiments conducted on different adenoma cells, dopamine at a concentration of 0.1  $\mu$ M significantly inhibited accumulation of ACTH in the culture medium. The extent of suppression by dopamine was similar; 61.0 (No. 1), 65.7 (No. 2), 60.5 (No. 3), 60.5 (No. 4, on day 5), and 71.5% (No. 5) of each control, respectively. A similar result was obtained when the same adenoma cells (No. 4) were exposed to 0.1  $\mu$ M dopamine on day 13 in culture, confirming that the inhibitory effect of dopamine on ACTH secretion from corticotroph adenoma cells is reproducible. Concomitant incubation with a dopaminergic antagonist, haloperidol, at an equimolar concentration resulted in a partial blockade of dopamine effect (Nos. 3 and 5). The addition of haloperidol at a higher concentration completely reversed the inhibitory effect of dopamine on ACTH secretion (No. 3).

*Effect of TRH and hydrocortisone on ACTH secretion from cultured adenoma cells.* The addition of TRH to the culture medium significantly stimulated secretion of immunoreactive ACTH. This effect was most clearly observed in one experiment using the pituitary adenoma cells obtained from a patient with Nelson's syndrome (patient 1) in whom TRH induced a marked elevation of plasma ACTH (Fig. 1). Moreover, a dose-response curve was obtained between the concentrations of TRH and ACTH response (Fig. 7). In the same adenoma cells hydrocortisone (1  $\mu$ M) caused

a significant ( $P < 0.05$ ) suppression of ACTH release (Fig. 7). An inverse dose-response relationship was observed between hydrocortisone and ACTH accumulation in the medium.

To examine the interaction between the stimulatory effect of TRH and the suppressive action exerted by cyproheptadine as well as by hydrocortisone on ACTH release, the following experiments were performed. The cultured cells from patient 2 also responded to 0.1  $\mu$ M TRH by secreting ACTH ( $P < 0.05$ ) (Fig. 8). When cyproheptadine was coincubated with TRH, this stimulatory effect of TRH was overcome. In adenoma cells from patient 3, TRH at a concentration of 0.1  $\mu$ M did not show any significant effect, while 0.1  $\mu$ M hydrocortisone inhibited ACTH release ( $P < 0.05$ ) (Fig. 8). When cells were incubated with TRH and hydrocortisone at an equimolar concentration, the inhibitory effect of hydrocortisone disappeared. Hydrocortisone at a higher concentration (1  $\mu$ M), however, effectively abolished the effect of TRH. TRH had no stimulatory effect on cultured cells from patient 5, which was consistent with the observation that TRH injection failed to elevate plasma ACTH concentrations in this patient (Fig. 1).

*Effect of drugs and hormones on ACTH secretion from cultured normal human corticotrophs.* To determine whether ACTH secretion from cultured cells in response to various stimuli described above is characteristic of the ACTH-producing pituitary adenoma cells, a nonadenomatous anterior pituitary tissue obtained from a patient at the time of hypophysectomy for palliation of metastatic breast cancer was similarly cultured. The effects of drugs and hormones on ACTH secretion from the cells in culture

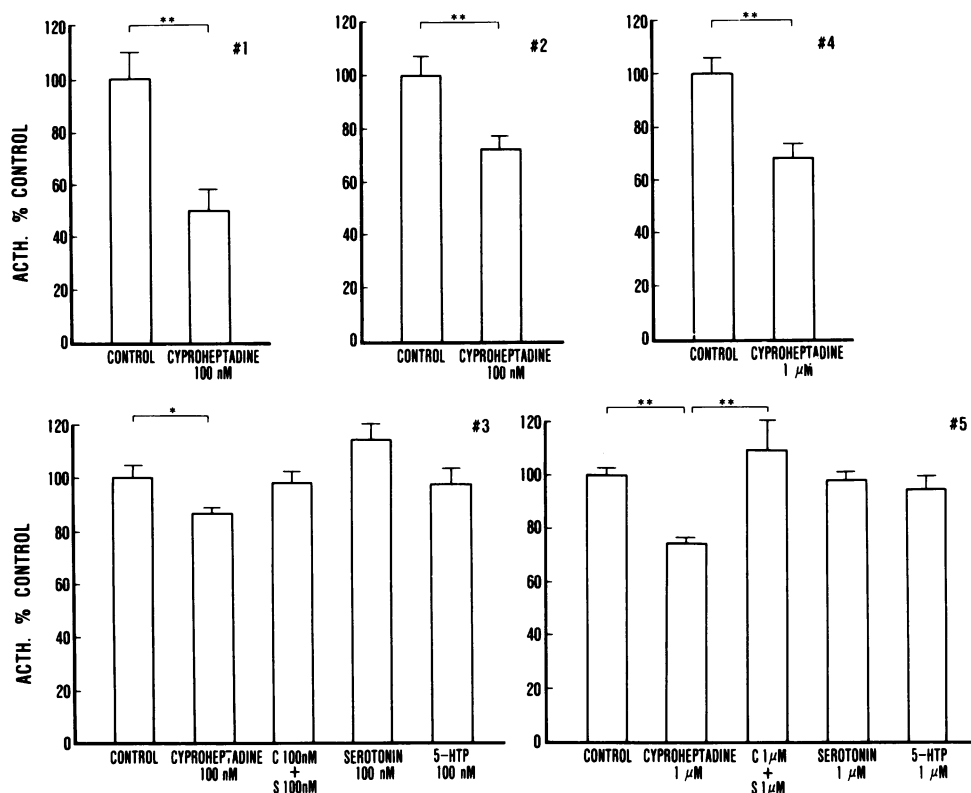


FIGURE 5 Effect of cyproheptadine, serotonin, and 5-hydroxytryptophan (5-HTP) alone or in combination on ACTH secretion by cultured corticotroph adenoma cells obtained from patients with Nelson's syndrome (patients 1-4) and a patient with Cushing's disease (patient 5). A minimum of four replicates was used for each variable. Results are expressed as the percentage of change in secretion relative to a preincubation in medium alone. For comparison, the mean value in the control incubation was designated as 100%. Results are the mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

is depicted in Fig. 9. The addition of hydrocortisone at a concentration of  $0.1 \mu\text{M}$  to the culture resulted in a significant decrease in ACTH release ( $P < 0.01$ ) by  $\sim 50\%$ . Conversely, incubation of cells with synthetic lysine vasopressin ( $0.1 \mu\text{M}$ ) stimulated the cultured corticotrophs to secrete ACTH significantly, compared with controls ( $P < 0.01$ ). In contrast, cells did not show any response to cyproheptadine, dopamine, and TRH at  $1 \mu\text{M}$ .

## DISCUSSION

Paradoxical or aberrant ACTH response to TRH has been recently described in patients with Cushing's disease and in those with Nelson's syndrome (29, 30). The mechanism and the site of action of TRH to induce ACTH release in this pathological condition are unknown. TRH may act on the hypothalamus, as suggested by Krieger et al. (8, 29); or alternatively TRH may possess a direct action on pituitary adenoma of these patients to stimulate ACTH secretion. On the basis of our previous findings that anomalous growth

hormone responses in acromegalics could be explained by the effect of TRH on pituitary tumors at a cellular level (21, 31), the latter explanation seemed to us to be more likely. We then examined the effect of TRH on ACTH secretion using cultured pituitary adenoma cells obtained from patients with Cushing's disease and Nelson's syndrome in the present study. In adenoma cells from two subjects whose plasma ACTH levels rose in response to TRH injection before surgery, a significant increase in ACTH secretion in vitro was observed. That this effect of TRH on ACTH release is merely an artifact is unlikely, since a dose-response relationship was observed in one experiment between TRH concentrations and the response of ACTH (Fig. 7). Moreover, adenoma cells from a patient with Cushing's disease (patient No. 5) who failed to show ACTH response to TRH in vivo, again did not respond to TRH in vitro. The observation that TRH was inactive in triggering ACTH release from normal human corticotrophs (Fig. 9) may also support our view. These results led us to the conclusion that TRH stimulates ACTH secretion in patients with Cushing's disease and

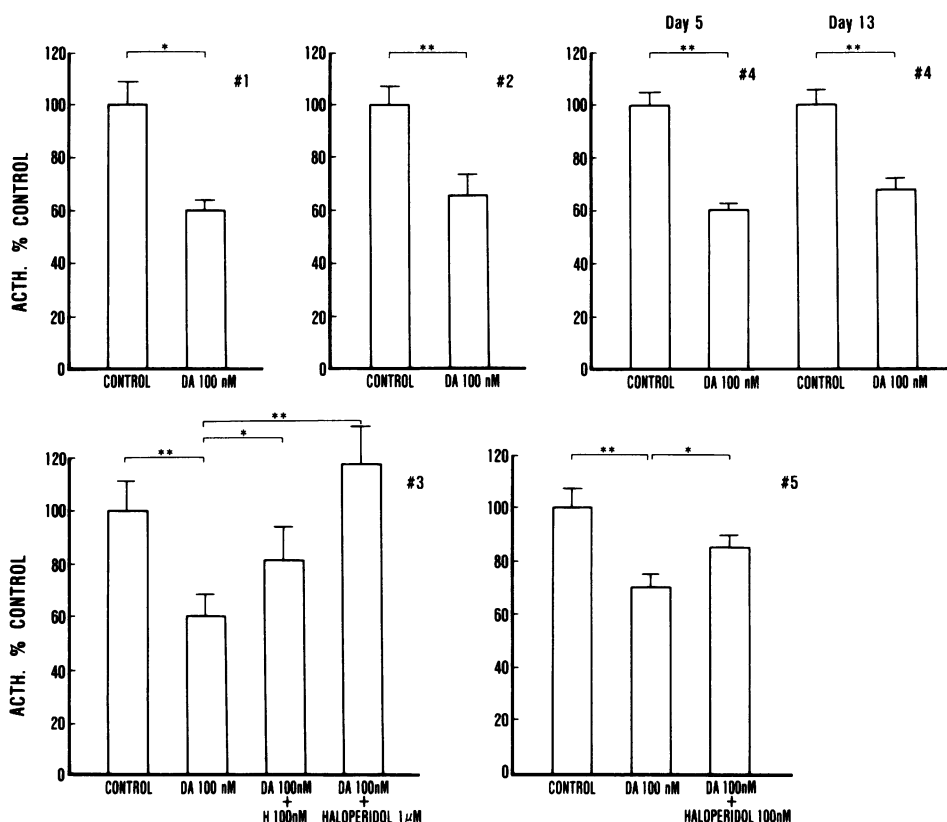


FIGURE 6 Effect of dopamine (DA) alone or in combination with haloperidol on ACTH secretion by cultured corticotroph adenoma cells obtained from patients with Nelson's syndrome (patients 1-4) and a patient with Cushing's disease (patient 5). In one adenoma tissue (No. 4), experiments were repeated on day 5 and on day 13 in culture to ascertain whether the response was reproducible. A minimum of four replicates was used for each variable. Results are expressed as the percentage of change in secretion relative to a preincubation in medium alone. For comparison, the mean values in the control incubation were designated as 100%. Results are the mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

Nelson's syndrome by a direct effect on ACTH-secreting pituitary adenoma, although the possibility that TRH may act both on the adenoma and on the hypothalamus could not be ruled out. Of relevance in this regard is the recent observation by Gershengorn et al. (32) that ACTH-producing mouse pituitary cells (AtT-20/NYU-1) possess the receptors for TRH, and that TRH stimulates both production and secretion of ACTH.

Evidence has been accumulated recently for a serotonergic mechanism involved in the regulation of ACTH secretion both in men and in experimental animals (33, 34). Serotonergic blockade by cyproheptadine, therefore, may inhibit ACTH secretion by hypothalamic inhibition in patients with Cushing's disease and in those with Nelson's syndrome. Altered central nervous system function was postulated in these patients (1, 29, 34). However, a significant decrease in immunoreactive ACTH released into the medium, compared with control incubation, was ob-

served when cyproheptadine was added to adenoma cells at a concentration of 0.1 or 1  $\mu$ M (Fig. 5). Furthermore, coincubation of cells with serotonin blocked this inhibitory effect of cyproheptadine, which may indicate that the action of this drug is mediated by its antiserotonergic property. In contrast, normal human corticotrophs did not show any response to cyproheptadine at 1  $\mu$ M (Fig. 9). These results suggest that cyproheptadine may exert a direct action on pituitary corticotroph adenoma to inhibit the hormone secretion. Favoring of this view is the clinical observation that cyproheptadine was effective in lowering ACTH levels in a patient with an ectopic ACTH-producing tumor (35). When TRH was coincubated with cyproheptadine, the stimulatory effect of TRH on ACTH release was overcome. Similarly, hydrocortisone at a higher concentration blocked TRH-mediated ACTH secretion (Fig. 8). This implies that the clinical effect of cyproheptadine in Cushing's disease and Nelson's syndrome may be occasioned by

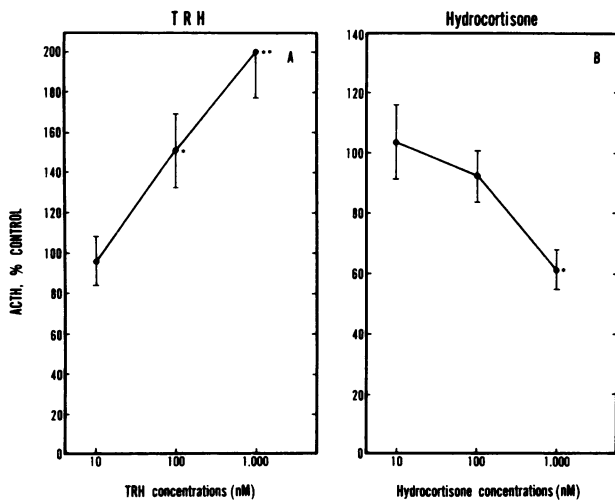


FIGURE 7 Effect of varying doses of TRH (A) or hydrocortisone (B) on ACTH secretion by cultured corticotroph adenoma cells obtained from a patient with Nelson's syndrome (patient 1). A minimum of four replicates was used for each variable. Results are expressed as the percentage of change in secretion relative to a preincubation in medium alone. For comparison, the mean values in the control incubation were designated as 100%. Results are the mean  $\pm$  SEM. \*,  $P < 0.05$  vs. control; \*\*,  $P < 0.01$  vs. control.

lowering the basal secretion of the hormone from corticotroph adenoma and, in addition, by blocking the ACTH release in response to secretagogues. Krieger and Kondon (8) observed in a patient with Nelson's syndrome that cyproheptadine treatment resulted in a lack of responsiveness of plasma ACTH levels to TRH administration, in contrast to the response seen in the untreated state. Serotonin by itself, on the other hand, failed to affect ACTH secretion by two adenoma cell cultures studied (Fig. 5), which confirms the recent observation of Mashiter et al. (27). Similarly, 5-hydroxytryptophan was also ineffective in this regard (Fig. 5). The reason why adenoma cells responded to cyproheptadine but not to serotonin remains unknown.

In addition to cyproheptadine, the therapeutic action of bromocriptine in Cushing's disease has been evaluated in recent years (9–15). A marked diminution of plasma ACTH after a single oral dose of bromocriptine has been observed in some patients with the disease as well as in subjects with Nelson's syndrome (9–12, 15), although other workers failed to demonstrate such an effect (13, 14). In the present study, 0.1  $\mu$ M dopamine inhibited the release of ACTH in all of the five adenoma cells studied. That this was simply caused by nonspecific action of dopamine is unlikely, since coinubation of cells with haloperidol resulted in the reversal of dopamine effect. Moreover, the inhibitory effect of dopamine on ACTH secretion was not observed in normal human corticotrophs in culture

(Fig. 9). Thus, it may be plausible to ascribe the effect of bromocriptine, a specific dopamine receptor agonist with a prolonged action, to a direct inhibition of ACTH release from corticotroph adenoma cells in these patients. Compared with the previous reports on the efficacy of bromocriptine in patients with Cushing's disease (9–15), the percentage of responders in the present study (five out of five pituitary adenomas) is high. However, it should be noted that four out of five adenomas were obtained from patients with Nelson's syndrome. The suppressive effect of bromocriptine on ACTH release is more frequently observed in patients with Nelson's syndrome than in those with Cushing's disease (15).

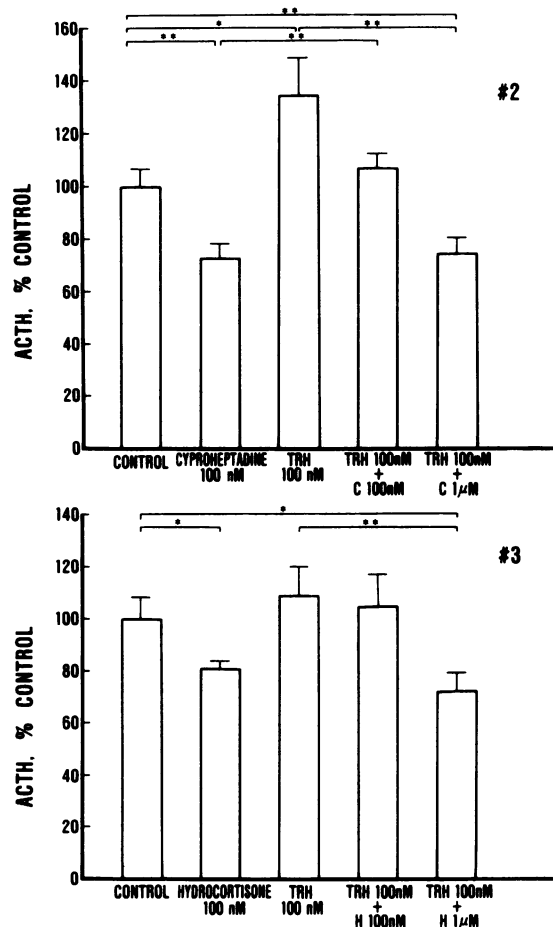


FIGURE 8 Effect of TRH, hydrocortisone, and cyproheptadine alone or in combination on ACTH secretion by cultured corticotroph adenoma cells obtained from patients with Nelson's syndrome (patients 2 and 3). A minimum of five replicates was used for each variable. Results are expressed as the percent change in secretion relative to a preincubation in medium alone. For comparison, the mean value in the control incubation was designated as 100%. Results are the mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



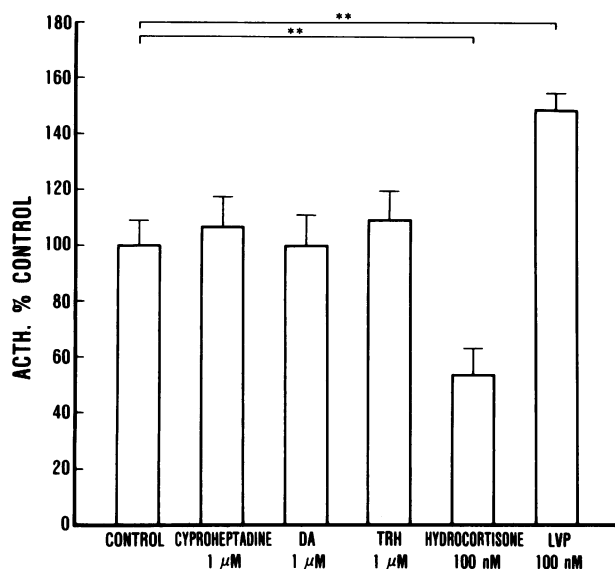


FIGURE 9 Effect of cyproheptadine, dopamine, TRH, hydrocortisone, and lysine vasopressin on ACTH secretion by cultured normal human corticotrophs obtained from a patient at the time of hypophysectomy for the palliation of the metastatic breast cancer. A minimum of four replicates was used for each variable. Results are expressed as the percentage of change in secretion relative to a preincubation in medium alone. For comparison, the mean value in the control incubation was designated as 100%. Results are the mean  $\pm$  SEM. \*\*,  $P < 0.01$ .

The direct action of various concentrations of hydrocortisone on corticotroph adenoma cells was studied. In one adenoma cell culture (No. 1), no significant suppression of ACTH release was demonstrated by  $0.1 \mu\text{M}$  hydrocortisone, however, a marked inhibition was observed at supraphysiological concentration of hydrocortisone ( $1 \mu\text{M}$ ) (Fig. 7). The addition of hydrocortisone at the lower concentration resulted in a modest but a significant decrease in ACTH secretion in the other (No. 3) (Fig. 8). These results suggest that hydrocortisone inhibits ACTH secretion by acting on adenoma cells of these patients. The site of corticosteroid-feedback control of ACTH in men has not been clarified, but a number of studies hitherto obtained in experimental animals favor the pituitary level (36–39). In fact, ACTH secretion from cultured normal human corticotrophs was markedly suppressed by hydrocortisone at the lower concentration ( $0.1 \mu\text{M}$ ). The abnormalities of steroid feedback regulation in Cushing's disease and in Nelson's syndrome, therefore, could be largely explained by altered putative steroid receptors on adenoma cells, which may be less sensitive when compared with those of normal corticotrophs.

The present study has provided the evidence for the ability of a direct action of cyproheptadine and dopamine, as well as hormones including TRH and hydrocortisone, on corticotroph adenomas to affect

ACTH secretion. The precise mechanism of the action of these substances, however, remains unknown. The fact that some patients respond to these stimuli while others do not is difficult to explain. Further studies may be necessary for the solution of these questions.

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