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Thyrotropin-releasing Hormone Stimulation of Adrenocorticotropin Production by Mouse Pituitary Tumor Cells in Culture: *POSSIBLE MODEL FOR ANOMALOUS RELEASE OF ADRENOCORTICOTROPIN BY THYROTROPIN-RELEASING HORMONE IN SOME PATIENTS WITH CUSHING'S DISEASE AND NELSON'S SYNDROME*

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POSSIBLE MODEL FOR ANOMALOUS RELEASE OF ADRENOCORTICOTROPIN BY THYROTROPIN-RELEASING HORMONE IN SOME PATIENTS WITH CUSHING'S DISEASE AND NELSON'S SYNDROME

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ABSTRACT ACTH-producing mouse pituitary tumor cells in culture (AtT-20/NYU-1 cells) were found to have binding sites for thyrotropin-releasing hormone (TRH). These putative receptors bound TRH with high affinity; the apparent equilibrium dissociation constant was 3.7 nM. The affinity of the receptors for a series of TRH analogues was similar to those previously reported for TRH-receptor interactions on thyrotropic and mammotropic cells in culture. Like some human pituitary tumors *in situ*, AtT-20/NYU-1 cells were found to produce the alpha subunit of the glycoprotein hormones (alpha). Alpha accumulation in the medium was constant (3.1 ng/mg cell protein per h) and was not affected by TRH. In contrast, TRH increased the amount of ACTH accumulated in the medium from AtT-20/NYU-1 cells to 190 and 420% of control at 1 and 24 h, respectively. TRH induced a dose-dependent increase in ACTH release during a 30-min incubation; half-maximal stimulation occurred at ~0.1 nM. TRH had no effect on ACTH release in vitro from anterior pituitary cells derived from normal rats. Because TRH stimulates release of ACTH in some untreated patients with Cushing's disease and Nelson's syndrome as well as pathological states associated with pituitary tumors (but not in normal subjects), AtT-20/NYU-1 cells may serve as an important in vitro model for human pituitary ACTH-secreting adenomas. Moreover, these findings suggest that the primary abnormality in Cushing's disease and Nelson's syndrome, allowing TRH stimulation of ACTH release, may be intrinsic to neoplastic adrenocorticotrophs rather than in neuroregulation of ACTH release.

INTRODUCTION

An anomalous stimulatory effect of thyrotropin-releasing hormone (TRH)[1] on release of ACTH has been described in some untreated patients with Cushing's disease and Nelson's syndrome (1, 2). It was suggested that this response may have been the result of either "altered function of neuroregulatory pathways involved in ACTH release" or to "alteration in hormone receptors associated with the neoplastic process" (1). To study whether the primary abnormality, allowing TRH stimulation of ACTH release, is intrinsic to neoplastic adrenocorticotrophs, it is necessary to employ cells that are removed from the influence of neuroregulatory factors. Previous reports have documented the usefulness of several cell culture systems derived from rodent tumors as models in which to study

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[1] *Abbreviations used in the paper*: TRH, thyrotropin-releasing hormone; TSH, thyrotropin; alpha, alpha subunit of the glycoprotein hormones; LH, luteinizing hormone; TRH-free acid, pGlu-His-Pro-OH.

regulation of pituitary hormone production by TRH (3–8). TRH stimulates prolactin and inhibits growth hormone production by clonal strains of rat pituitary tumors (GH cells) and stimulates thyrotropin production by pituitary tumor cells derived from mice of the LAF_1 strain (TtT cells). Furthermore, TtT cells exhibit certain functional parallels to human pituitary thyrotropic tumors which are not found in normal or hyperplastic pituitary glands, such as excess production of the alpha subunit of the glycoprotein hormones (alpha) (7). An ACTH-producing cell line (AtT-20) derived from a pituitary tumor from an irradiated LAF_1 mouse (9) has been employed in a series of studies of ACTH synthesis and secretion (10–16). We examined the interaction of TRH with a newly generated subpopulation of AtT-20 cells (AtT-20/NYU-1) and its effect on ACTH release to determine whether TRH may stimulate ACTH release directly from neoplastic cells.

In this report we demonstrate that AtT-20/NYU-1 cells possess putative receptors for TRH, produce alpha subunit, and are stimulated to release ACTH by TRH. Release of ACTH from adenohypophyseal cells in vitro derived from normal rats, in contrast, is not affected by TRH. These data show that neoplastic mouse adrenocorticotrophs respond to TRH and suggest that the anomalous response of ACTH to TRH in patients with Cushing's disease and Nelson's syndrome may be caused by an alteration intrinsic to the adrenocorticotroph and not to changes in neuroregulation of ACTH release.

METHODS

[^{3}H]TRH (L-[2,3,4,5-^{3}H]proline, 115 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Unlabeled TRH and TRH-free acid (pGlu-His-Pro-OH) were from Beckman Instruments, Inc., Fullerton, Calif., pGlu-3-MethylHis-Pro-NH_2 was from Calbiochem, San Diego, Calif., and pGlu-His-Pro-$NHCH_2CH_3$ was generously provided by Dr. E. D. Nicolaides of Parke, Davis & Company, Detroit, Mich. Dexamethasone was from Sigma Chemical, Co., St. Louis, Mo. Ham's F-12 medium and horse and fetal bovine sera were obtained from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.

ACTH was measured by radioimmunoassay (17, 18) using reagents kindly provided by the National Pituitary Agency, National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD), Baltimore, Md. The antiserum (West, batch 2) cross-reacts with the α (11–24) region of ACTH (19). Synthetic human α 1–39 ACTH was used for iodination and as standard. Alpha (7), thyrotropin (TSH) (7, 8, 20), and prolactin (21) were measured by radioimmunoassays using reagents for rat hormones from the NIAMDD Hormone Distribution Program (Dr. A. F. Parlow) as previously described.

AtT-20 cells were obtained from the American Type Culture Collection, Rockville, Md., and grown in Ham's F-12 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (9). After several weeks in culture, a subpopulation of cells developed (AtT-20/NYU-1) that grew as monolayers as well as in suspension culture, similar to the previously described AtT-20/D-16 cells (15, 16). These cells, when incubated in medium with serum, released 0.20±0.06 μg of ACTH/mg cell protein per 24 h. There was no detectable immunoreactive prolactin (<8 ng/mg cell protein per 24 h) or TSH (<7 ng/mg protein per 24 h) in the incubation medium.

Binding of TRH to intact cells in suspension was performed in fresh medium without serum containing [^{3}H]TRH at 37°C in a shaking water bath (8). Experiments were carried out in 0.5 ml of medium in 12 × 75-mm glass test tubes with 0.5–2 × 10^6 cells. After incubation the cells were centrifuged at 650 *g* for 5 min at 4°C, the medium was removed, and the cells were washed three times with 1 ml of 0.1 M NaCl, 0.05 M Na phosphate, pH 7.5. The cell pellets were dissolved in 0.4 N NaOH; portions were used to measure radioactivity by liquid scintillation counting and for measurement of cell protein by the method of Lowry et al. (22) using bovine serum albumin as standard. Nonspecific binding, that is [^{3}H]TRH bound in the presence of a 200-fold excess of unlabeled TRH, was subtracted from each value. Nonspecific binding represented <15% of total bound radioactivity.

Incubation medium without serum from AtT-20/NYU-1 cells was pooled and analyzed in the alpha radioimmunoassay at several dilutions. A 4-ml portion was lyophilized, resuspended in 0.5 ml water, and co-chromatographed with trace amounts of rat [^{125}I]iodo-TSH, rat [^{125}I]iodo-luteinizing hormone (LH)-alpha, and ^{125}I on a Sephadex G-100 column (90 × 1.5 cm). The column was equilibrated at 4°C with 0.1 M NaCl, 0.05 M Na phosphate, pH 7.5, and eluted at a flow rate of 4 ml/h. Portions of each 1-ml fraction were assayed for alpha.

ACTH and alpha accumulation in the medium from AtT-20/NYU-1 cells was determined at various times of culture in the presence of 100 nM TRH or 100 nM dexamethasone and compared to controls. For these experiments, 0.5–2 × 10^6 cells were inoculated in F-12 medium with serum. After 16–24 h, the cell monolayers (in flasks) or cells in suspension (in roller bottles) were washed and fresh F-12 medium with or without serum and TRH or dexamethasone was added and the incubation continued. At the indicated times, media were collected and stored at −20°C until assayed and the cells washed three times with 0.1 M NaCl, 0.05 M Na phosphate, pH 7.5, dissolved in 0.4 N NaOH and used for measurement of cell protein.

Primary monolayer cultures of rat adenohypophyseal cells were prepared by the method of Vale et al. (23) with minor modification. In brief, anterior pituitary glands were removed from male Sprague-Dawley rats (200–250 g) after rapid decapitation, rinsed five times in 0.1 M NaCl, 0.05 M Na phosphate, pH 7.4, 0.01 M glucose, 0.36 mM $CaCl_2$, and then cut into small pieces. The pituitary fragments were then suspended in the same buffer containing 0.1% hyaluronidase (type II, 200 NF U/mg; Sigma Chemical Co.) and 0.35% collagenase (160 U/mg; Sigma Chemical Co.) and incubated at 37°C with stirring. After 30 min, a solution containing pancreatin 4X (Grand Island Biological Co.), final concentration 0.25%, was added to the dispersion mixture. After an additional 15 min, the dispersed cells were washed once with F-12 medium with serum, resuspended in the same medium, and 1 × 10^6 cells inoculated into 25-cm^2 culture dishes. After 48 h, the primary cell monolayers were washed with F-12 medium without serum and incubated in parallel in F-12 medium without serum with AtT-20/NYU-1 cells. Release of ACTH from adenohypophyseal cells in culture was found to increase 1.4- to 2-fold during a 1-h exposure to 10 nM vasopressin as previously described (24, 25) demonstrating that these cells were responsive to a secretagogue.

Statistical analysis was performed by unpaired *t* test. For the alpha radioimmunoassay, the plots of the standard curve and of the curve generated with dilutions of incubation

medium were determined by linear regression analysis and parallelism was determined by analysis of covariance (26).

RESULTS

Binding of TRH and analogues to AtT-20/NYU-1 cells. [^{3}H]TRH added to the medium of suspensions of AtT-20/NYU-1 cells bound rapidly to the cells. Specific binding was 30% of maximum after 5 min, 50% after 15 min, 86% after 45 min, maximum after 60 min, and remained constant for up to 3 h. The characteristics of binding of TRH at equilibrium were defined by incubating cells with increasing concentrations of [^{3}H]TRH, up to 55 nM, for 90 min. Fig. 1 illustrates the dose-dependent specific binding of [^{3}H]TRH to AtT-20/NYU-1 cells. Analysis of these data by the method of Scatchard (27) (Fig. 1, inset) yields a linear plot consistent with a single class of noninteracting binding sites. The dissociation constant was 3.7 nM. There were 28 fmol [^{3}H]TRH bound/mg cell protein at saturation.

The relative affinities of the binding sites for several TRH analogues were determined by comparing the ability of the analogues to compete with [^{3}H]TRH for binding at equilibrium. Table I shows the dissociation constants of the analogues based on the value measured for TRH (from Fig. 1). The affinity of the binding sites for p-Glu-3-MethylHis-Pro-NH_2 was higher than for TRH but was markedly lower for the other analogues.

Release of alpha from AtT-20/NYU-1 cells. Fig. 2A illustrates the standard curve using purified rat LH-alpha and a curve generated using dilutions of pooled incubation medium for the alpha radioimmunoassay. Medium in which cells had not been incubated had no detectable alpha immunoactivity. Immunological similarity between alpha in the medium and rat LH-alpha was suggested by parallelism ($P > 0.1$) between these curves. Fig. 2B illustrates the gel chromatographic elution pattern of alpha in the incubation medium.

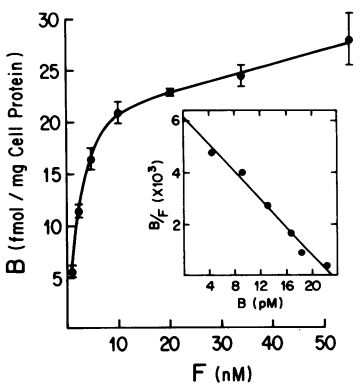

FIGURE 1 [^{3}H]TRH binding to AtT-20/NYU-1 cells at equilibrium. 2×10^6 cells were incubated with [^{3}H]TRH, 1–55 nM, for 90 min after which bound [^{3}H]TRH was measured. The points represent the mean±SD of triplicate determinations. (Inset) Plot of these data by the method of Scatchard (27).

TABLE I
*Binding Affinities of TRH Analogues**

	Dissociation constant
	nM
TRH	3.7
pGlu-3-MethylHis-Pro-NH_2	0.41
pGlu-His-Pro-$NHCH_2CH_3$	93
TRH-free acid	110,000

* 2×10^6 cells in suspension in F-12 medium without serum were incubated for 2 h with 10 nM [^{3}H]TRH and various concentrations of unlabeled TRH analogues. The dissociation constant of TRH was derived from the data in Fig. 1. The dissociation constants of the analogues were determined by comparing the dose-response curves of their inhibition of [^{3}H]TRH binding to that of TRH.

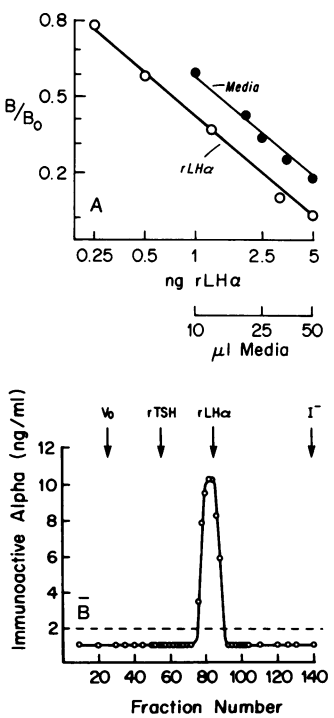

FIGURE 2 Immunologic and chromatographic similarity of alpha released from AtT-20/NYU-1 cells and rat LH-alpha. (A) Displacement curve of incubation medium in the alpha radioimmunoassay. Curve was generated with dilutions of pooled incubation medium which did not contain serum. (B) Gel chromatography on a (1.5 × 90-cm) column of Sephadex G-100 of pooled incubation medium. Fractions were assayed in the alpha immunoassay. Recovery of alpha immunoactivity was >95%. Elution positions of rat [^{125}I]iodo-TSH, rat [^{125}I]iodo-LH-alpha, and ^{125}I$^-$, used as internal column markers, and V_0 are shown.

Virtually all alpha immunoactivity eluted in a position similar to that of standard rat [^{125}I]iodo-LH-alpha.

Fig. 3 illustrates alpha accumulation in the medium from AtT-20/NYU-1 cells in the presence of 100 nM TRH or in its absence (control). The rate of alpha accumulation was constant during the 72-h incubation (3.1 ng/mg cell protein per h). There was no effect of TRH on alpha accumulation. Furthermore, 100 nM dexamethasone, which inhibited ACTH accumulation from these cells (see below), had no effect on accumulation of alpha during a 24-h incubation (Table II).

Effect of TRH on accumulation of ACTH in the medium. 100 nM dexamethasone inhibited accumulation of ACTH by 44% from AtT-20/NYU-1 cells after 24 h (Table II). These findings are similar to those reported for other cell lines derived from this tumor (10, 15). TRH stimulated production of immunoreactive ACTH by these cells. In one experiment (Table III), the amount of ACTH accumulated in the medium was increased to 260% of control after 24 h. The increase of immunoreactive ACTH by TRH was not caused by stabilization of ACTH in the medium because TRH did not affect the recovery of exogenously added standard ACTH. Fig. 4 illustrates the dose-dependent stimulation of ACTH release by TRH after 30 min. Half-maximal stimulation occurred at ~0.1 nM. TRH stimulation of accumulation of ACTH from AtT-20/NYU-1 cells was demonstrable from cells incubated in medium without serum but not in the presence of serum. In another experiment, basal ACTH release into medium without serum was increased to 190% of control after 1 h by 100 nM TRH (Table III). In contrast, release of ACTH from cells incubated in medium with serum but without TRH was 240±80% of the serum-free control ($P < 0.05$) and there was no further increase with exposure to TRH (220±53% of serum-free control; $P > 0.1$ vs. medium with serum alone). A further stimulation of ACTH accumulation by TRH into medium without serum, to 420% of control, occurred after 24 h.

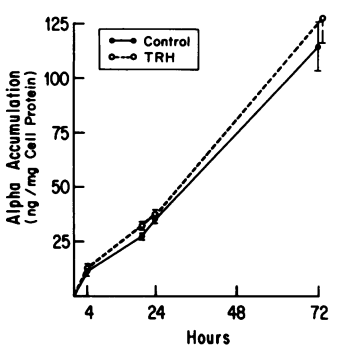

FIGURE 3 Accumulation of alpha in the medium from AtT-20/NYU-1 cells in the presence of TRH and in its absence. 0.5 × 10^6 cells were inoculated into dishes in F-12 medium with serum. After 16 h, the medium was removed and replaced with fresh F-12 medium without serum with no addition (control) or with 100 nM TRH. Points represent mean±SD of triplicate determinations harvested at the indicated times.

TABLE II

*Effect of TRH and Dexamethasone on Accumulation of ACTH and Alpha in the Medium after 24 h**

	ACTH	Alpha
	ng/mg cell protein	
Control	27±0.73	38±3.8
Dexamethasone	15±3.8‡	32±4.6§
TRH	70±13‖	35±4.3§

* 1 × 10^6 cells were inoculated in F-12 medium with serum. After 16 h, the cells were washed and incubated with fresh F-12 medium alone (control) or with 100 nM dexamethasone or 100 nM TRH for 24 h.
‡ $P < 0.005$ vs. control.
§ NS vs. control.
‖ $P < 0.001$ vs. control.

In contrast to the findings in AtT-20/NYU-1 cells, TRH had no effect on accumulation of ACTH from adenohypophyseal cells in vitro derived from normal rats after 1 or 24 h in F-12 medium without serum (Table III).

DISCUSSION

An ACTH-producing mouse pituitary tumor cell line (AtT-20) has been effectively employed in a series of

TABLE III

Effect of TRH on Accumulation of ACTH in the Medium from AtT-20/NYU-1 Cells and Adenohypophyseal Cells Derived from Normal Rats

	ACTH	
	Control	TRH
	ng/mg cell protein	
AtT-20/NYU-1 cells*		
1 h	14±1.5	26±3.5
24 h	23±2.3	97±16
Adenohypophyseal cells‡		
1 h	55±10	55±6.1
24 h	150±19	140±13

* 1 × 10^6 cells were inoculated in F-12 medium with serum. After 96 h, the cells were washed and incubated in fresh medium without serum alone (control) or with TRH (100 nM). For AtT-20/NYU-1 cells, TRH vs. control: 1 h, $P < 0.01$; 24 h, $P < 0.005$.
‡ 0.5 × 10^6 acutely dispersed cells were inoculated in F-12 medium with serum. After 48 h, the cells were washed and incubated in fresh medium without serum alone (control) or with 100 nM TRH. For adenohypophyseal cells, TRH vs. control: 1 h, $P > 0.1$; 24 h, $P > 0.1$.

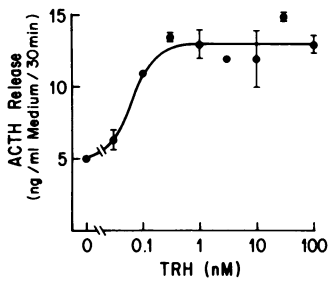

FIGURE 4 Effect of TRH on ACTH release from AtT-20/NYU-1 cells. Cells from suspension culture were resuspended in fresh medium without serum (2×10^6 cells/ml) and incubated with varying concentrations of TRH for 30 min. Points represent mean±SE of triplicate determinations.

studies of the secretion and synthesis of ACTH (10–16). In the experiments reported here, the interaction of TRH with a newly generated subpopulation of these cells (AtT-20/NYU-1) was studied. Receptors for TRH were demonstrated on these cells. The affinities of binding of these sites for a series of TRH analogues correlated with their known biological activities in other cell lines (5, 28, 29). AtT-20/NYU-1 cells were found also to produce the alpha subunit of the glycoprotein hormones but not intact TSH. Accumulation of alpha in the medium was not affected by TRH in these cells. In contrast to its lack of effect on alpha production, TRH increased accumulation of ACTH in the medium from AtT-20/NYU-1 cells. Stimulation of ACTH accumulation by TRH was shown to be specific to the neoplastic adrenocorticotroph and not a general phenomenon of ACTH-producing cells in culture, because TRH had no effect on accumulation of ACTH from cells in vitro derived from anterior pituitaries of normal rats. There are two explanations for this finding. First, the lack of effect of TRH on ACTH release from non-neoplastic adenohypophyseal cells may be the result of an absence of receptors for TRH in these cells. This can not readily be determined because adrenocorticotrophs comprise <11% of the total population of cells in the pituitary gland in which at least two other cells, thyrotrophs and mammotrophs, possess these receptors. Second, it is possible that there is a species difference between mouse and rat and that TRH may stimulate ACTH release from non-neoplastic cells derived from mice. This appears less likely because regulation of ACTH secretion in these rodents is similar. Thus, TRH directly stimulated release of ACTH from neoplastic but not from non-neoplastic adrenocorticotrophs in culture and this response was likely initiated by interaction with the demonstrated binding site for TRH.

Receptors for TRH have previously been demonstrated on mammotropic (GH) cells, cloned cell lines derived from rat pituitary tumors (5, 30–32), and thyrotropic (TtT) cells derived from mouse pituitary tumors (8, 29) in culture. The characteristics of the interaction between TRH and the binding sites on TtT, GH, and AtT-20/NYU-1 cells were very similar. The apparent equilibrium dissociation constants for binding of TRH were 10–12 nM for GH cells, 3–5 nM for TtT cells, and 3.7 nM for AtT-20/NYU-1 cells. Also, the relative affinities for a series of TRH analogues paralleled one another. Although determination of the binding affinity for hormone-receptor interactions is somewhat imprecise, we consistently measured a two- to threefold higher affinity for TRH binding to TtT and AtT-20/NYU-1 cell sites than to receptors on GH cells using the same [^{3}H]TRH preparation. Moreover, the virtually identical affinities of binding for TRH to TtT and AtT-20/NYU-1 cells may indicate that these receptor units are identical because these cell types were both derived from pituitary tumors from the same strain of mice (9, 33). The number of binding sites per cell, in contrast to the affinity of TRH binding, appeared to be different for AtT-20/NYU-1 cells compared to TtT and GH cells. Assuming a homogeneous population of cells, there were only 3,300 available receptor sites per AtT-20/NYU-1 cell whereas there were 99,000 (8) and 54,000–130,000 (30, 32) receptors per TtT and GH cell, respectively, in control cultures. The lower concentration of receptors on the ACTH-producing cells may reflect the absence of receptors on adrenocorticotrophs under normal physiological conditions, in contrast to their presence on normal thyrotrophs and mammotrophs. However, we cannot exclude, at this time, the possibility that the AtT-20/NYU-1 cultures are heterogeneous and that only a fraction of these cells, perhaps 5%, have receptors for TRH. In any event, we have demonstrated receptors for TRH on adenohypophyseal cells in culture that do not produce prolactin or TSH.

Secretion of the alpha subunit of the glycoprotein hormones by human pituitary tumors *in situ* has been demonstrated (34, 35). Measurement of alpha in serum has been suggested by us as one criterion for differentiating patients with pituitary tumors from those in which there is no evidence of a tumor in patients with TSH-induced hyperthyroidism (35, 36). Similar to the findings reported herein that AtT-20/NYU-1 cells produce alpha, we have shown previously that TtT cells produce excessive amounts of alpha (7). The immunologic and chromatographic properties of alpha from AtT-20/NYU-1 and TtT cells were indistinguishable from those of standard rat alpha. Thus, it appears that free alpha subunit may be produced by several different types of pituitary tumors and that these

mouse pituitary tumors may be exellent in vitro models for human pituitary adenomas. The finding that alpha production by AtT-20/NYU-1 cells was not increased by TRH is a further parallel to the findings in human pituitary tumors (36). If these cultures were heterogeneous (see above), a possible explanation for the lack of alpha response to TRH may be that the population of cells that produces alpha does not have receptors for TRH and, therefore, is unable to respond to TRH.

TRH caused an increase in release of immunoreactive ACTH from AtT-20/NYU-1 cells but not from adenohypophyseal cells in vitro derived from normal rats. The finding of no effect of TRH on release of ACTH from cells derived from normal anterior pituitary glands complements a previously published report using cells in vitro derived from rat pars intermedia (37) and the findings in normal human subjects (1). The ability of TRH to stimulate ACTH release from neoplastic but not "normal" cells in vitro appears to parallel the findings in man since it is likely that Nelson's syndrome and some instances of Cushing's disease are associated with pituitary adenomas (38). Although there may be additional neuroregulatory factors that influence the ability of TRH to affect ACTH release, as suggested by the study of Krieger and Condon (2), the primary abnormality in these clinical syndromes may be intrinsic to the ACTH-producing cell as with AtT-20/NYU-1 cells. Thus, neoplastic human as well as mouse ACTH-producing cells may synthesize receptors for TRH which are coupled to ACTH release and allow direct stimulation by TRH.

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