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APPARENT "NEGATIVE COOPERATIVITY" KINETICS IN THE ABSENCE OF A NONLINEAR SCATCHARD PLOT OF THYROTROPIN-RECEPTOR INTERACTION IN A HUMAN THYROID ADENOMA

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SUMMARY: A human thyroid adenoma (benign nodule) was identified which exhibited a linear Scatchard plot of ^{125}I-TSH binding, characteristic of a single class of binding site with high affinity (K_d = 0.5±0.1 nM) and low binding capacity (0.8±0.2 pmol/mg protein). In contrast, Scatchard analysis of binding to adjacent normal thyroid was nonlinear, suggesting the presence of high and low-affinity binding sites with K_d's of 0.4±0.2 and of 27.9±11.0 nM and capacities of 0.7±0.3 and 1.8±1.0 pmol/mg protein, respectively. Dissociation of bound ^{125}I-TSH from membranes of both adenoma and normal tissue revealed identical enhancement of dissociation in the presence of excess native hormone, thought to be evidence for the "negative cooperativity" model of hormone-receptor interaction. Furthermore, adenylate cyclase from both tissues was equally responsive to TSH. Thus, a thyroid adenoma which contains TSH-responsive adenylate cyclase still exhibited enhanced dissociation by native hormone, even though Scatchard analysis yielded a single, non-cooperative class of binding sites. This suggests that enhanced dissociation of bound hormone does not provide a demonstration of negatively-cooperative site-site interaction. Furthermore, nonlinear Scatchard plots, typical of TSH binding in normal thyroid, represent two classes of binding sites, of which the high affinity type is responsible for stimulation of adenylate cyclase.

INTRODUCTION: Equilibrium saturation analysis of thyrotropin (TSH) binding to thyroid plasma membranes (1-7), like some other peptide hormones (8-12), results in a nonlinear Scatchard plot. The nonlinear Scatchard plot was initially ascribed to multiple classes of binding sites, with different affinities for hormone. De Meyts et al. (8,9) proposed that this could also result from negatively cooperative site-site interaction among one homogeneous population of receptors. According to this model, "negative cooperativity" kinetics are demonstrated if the dilution-induced dissociation of receptor bound radioactive hormone is enhanced by excess native hormone in the dissociating medium (3,8,9), referred to here as "dissociation enhancement by cold hormone" (DEC)**. However,

**Abbreviations: Gpp(NH)p, 5'guanylimidodiphosphate; DEC, dissociation enhancement by cold hormone; Q_o, binding capacity.

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several laboratories including our own have suggested that the kinetic criteria upon which the negative cooperativity model largely depends are inadequate to accomodate numerous features of nonclassical binding of several peptide hormones (6,7,10-12).

Our previous studies showed that DEC is not a valid demonstration of negative cooperativity, since it occurred even when most of the available sites were occupied by hormone (6). Furthermore, DEC is not limited to thyroid membranes which exhibit nonlinear Scatchard plots; it is also observed in both target and non-target tissues under certain conditions in which the Scatchard plot was linear (7). However, these latter conditions were not physiological (pH 6.0), and therefore did not reflect true "receptor" binding. In this report, we identify a benign, non-functioning (as determined by uptake of technetium-99 in vivo) thyroid adenoma which exhibits a linear Scatchard plot at pH 7.4, indicating only one class of binding sites with apparent high affinity, in contrast to adjacent normal thyroid tissue which yields a nonlinear plot under identical assay conditions. This tissue was used to further evaluate the negative cooperativity model by examining the kinetic properties of TSH binding as well as the adenylate cyclase responsiveness to hormone.

MATERIALS AND METHODS: Specimens of adenoma and adjacent normal human thyroid were obtained at operation, and immersed immediately in liquid nitrogen. Partially purified plasma membranes were prepared as previously described (6). Purified bovine TSH (40 I.U./mg) was a gift of Dr. John Pierce (UCLA), and was iodinated using lactoperoxidase (7). Radiolabeled and radioinert TSH have equivalent affinities for binding sites on thyroid membranes (data not shown). In addition, the binding activity of ^{125}I-TSH (as determined by fraction of tracer bound by excess membranes) was 40% in both normal and adenoma thyroid.

Equilibrium binding analysis was performed by incubating 20 µg membrane protein with increasing concentrations of hormone, in 50 mM Tris-acetate buffer, 0.25% BSA, as detailed in figure legends. Bound hormone was separated from free on Millipore cellulose acetate filters, and corrections were made for filter and nonspecific binding (7). Binding data were analyzed according to the method of Scatchard (14), using the SCATFIT Computer Program (15).

Dissociation of ^{125}I-TSH was followed as previously described (7). Membranes were preequilibrated with ^{125}I-TSH and dissociation was induced by 100 fold dilution in the presence or absence of 10^{-8} M unlabeled TSH. Bound radioactivity was separated from free by filtration and expressed as percentage of bound hormone at time (t) = 0 min.

Adenylate cyclase activity was assayed by conversion of α^{32}P-ATP to cAMP in a final volume of 0.1 ml 50 mM Tris-acetate buffer, pH 7.6, for 15 min, at 30°C. 60 µg of membrane protein were added to a reaction medium containing 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM 3-isobutyl 1-methyl xanthine, 0.5 mM dithiothreitol, and an ATP regenerating system containing 5 mM phospho(enol)pyruvate, 10 U/ml pyruvate kinase, and 0.2 mM ATP. cAMP was separated on neutral alumina (16).

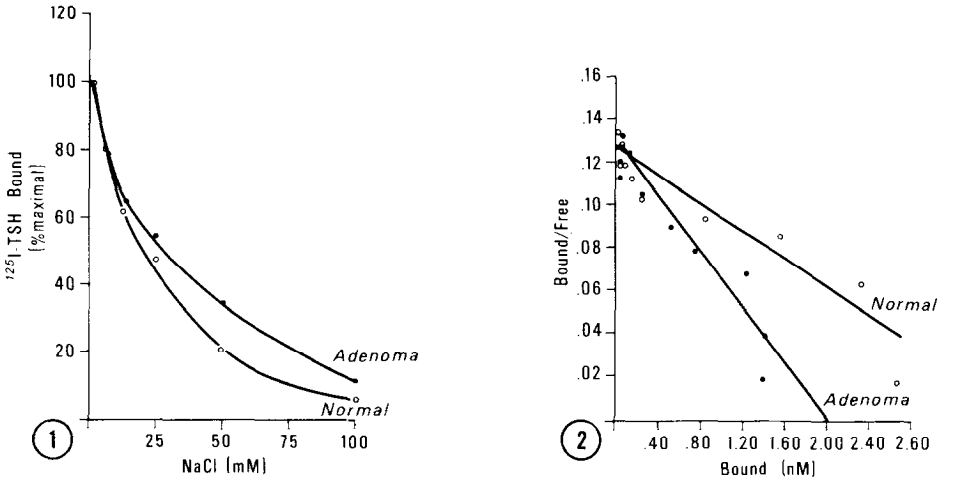

Fig. 1. Effect of salt on binding of ^{125}I-TSH. Membranes from adenoma (●) and adjacent normal thyroid (○) (40 µg/ml) were incubated with ^{125}I-TSH (30 pM) in 50 mM Tris-acetate buffer, 0.25% BSA, pH 7.4, for 1 h at 22°C, in the presence of varying concentrations of NaCl. The results are the means of triplicate determinations, expressed as a percentage of binding in the absence of NaCl.

Fig. 2. Scatchard plot of ^{125}I-TSH binding at pH 6.0. Membranes from adenoma (●) and adjacent normal thyroid (○) (40 µg/ml) were incubated in 50 mM Tris-acetate buffer 0.25% BSA pH 6.0, for 1 h at 22°C, with 20 pM ^{125}I-TSH and varying concentrations of unlabeled TSH. Data were analyzed by the SCATFIT computer programs; both adenoma and normal tissue were found to have one binding component. The data were not corrected to account for specific binding activity of the radiolabeled hormone (40%), however, identical binding activities were obtained in both tissues. Details in text.

RESULTS AND DISCUSSION: The binding of ^{125}I-TSH to membranes of adenoma and adjacent normal thyroid tissue was equally inhibited by the inclusion of increasing amounts of NaCl into the incubation medium (Fig. 1). The ID50 for NaCl inhibition of binding was approximately 25 mM, representing a total ionic strength significantly below physiological levels. This effect is typical of TSH radioligand-receptor binding in other species (1-7).

Binding in both tissues was maximal at pH 6.0. The physiological significance of TSH binding at this pH is not clear. However, it is unlikely to represent the biologically relevant TSH receptor, in view of recent studies showing that TSH binding at pH 6.0 lacks hormone (5) and tissue specificity (7), as well as experiments demonstrating the inability of TSH to stimulate adenylate cyclase under these conditions (data not shown). Parenthetically, we have recently found that binding of ^{125}I-TSH to talc occurs maximally at pH 6.0

(data not shown), suggesting that binding at this pH may be a property of the hormone and not the receptor.

Equilibrium saturation analysis of ^{125}I-TSH to thyroid membranes of both adenoma and normal tissue revealed linear Scatchard plots at pH 6.0, indicating that a single class of binding sites exists under these conditions (Figure 2). SCATFIT analysis of Scatchard plots yielded an apparent equilibrium dissociation constant (K_d) of 40±6 nM, and capacity (Q_o) of 55±8 pmol/mg protein for normal tissue and 16±2 nM and 25±2 pmol/mg protein for thyroid adenoma. In contrast, at pH 7.4 the binding profile of the thyroid adenoma was markedly different from that of adjacent normal gland (Figure 3). Whereas, Scatchard analysis of normal thyroid binding revealed two classes of binding sites with K_d's of 0.4±0.2 and 27.9±11.0 nM, and Q_o's of 0.7±0.3 and 1.8±1.0 pmol/mg protein, the adenoma yielded a linear Scatchard plot with K_d (0.5±0.1) and Q_o (0.8±0.2) values similar to those of the high affinity site of the normal gland.

The assumption that "dissociation enhancement by cold hormone" (DEC) occurs only in the presence of site-site interaction (nonlinear Scatchard plot)

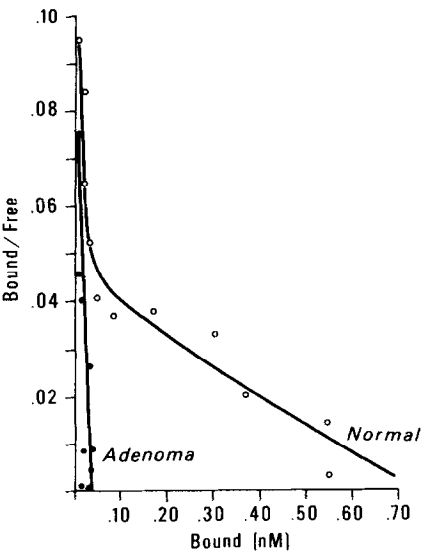

Fig. 3. Scatchard plot of ^{125}I-TSH binding at pH 7.4. Membranes from adenoma (●) and adjacent normal thyroid (○) were incubated as described in Fig. 2, at pH 7.4. SCATFIT analysis resolved the Scatchard plot of normal membranes into 2 binding components, and that of the adenoma into one binding component. Details in text.

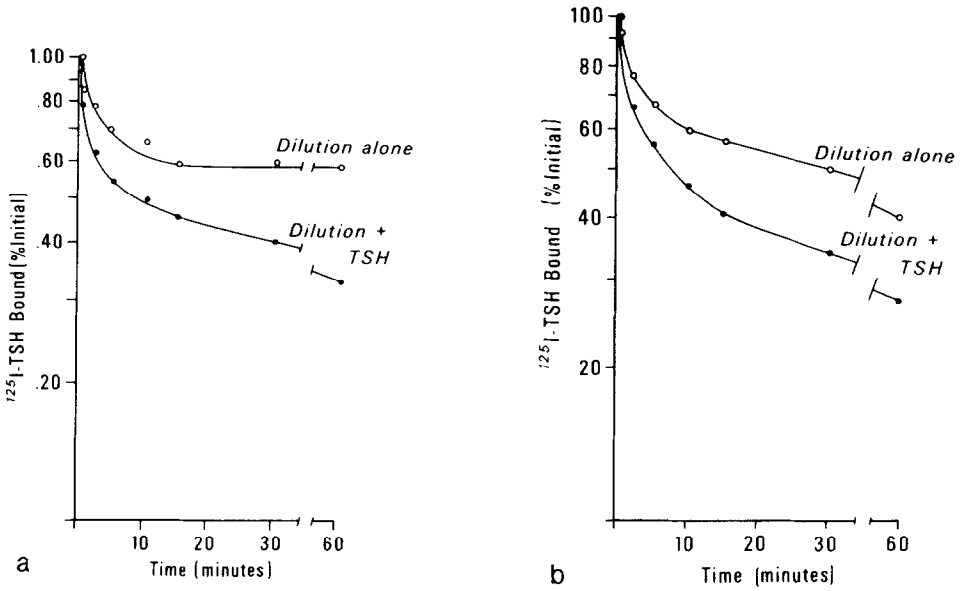

Fig. 4. Time course of dissociation of bound ^{125}I-TSH. Membranes from (a) adenoma and (b) adjacent normal thyoid (100 µg) were incubated with ^{125}I-TSH for 1 h at 22°C in 50 mM Tris-acetate buffer, 0.25% BSA, pH 7.4. Bound was separated from free hormone by centrifugation and resuspension in equal volume of buffer. 10 µl aliquots were diluted (1:100) in the presence (●) or absence (○) of 10 nM unlabeled TSH and bound radioactivity was allowed to dissociate at 22°C. Results are the means of triplicate determinations, expressed as a percentage of ^{125}I-TSH bound at t=0 min.

was central to the negative cooperativity model of De Meyts et al. (8,9). Supportive evidence had originally come from experiments describing growth hormone binding to human lymphocytes in which linear Scatchard plots were accompanied by the absence of DEC, and therefore, assumed not to be negatively cooperative (8). To test the validity of this assumption, membranes prepared from adenoma and adjacent normal thyroid were equilibrated with ^{125}I-TSH at pH 7.4, and membrane bound radioactivity was allowed to dissociate at 1:100 dilution in the presence or absence of excess unlabeled TSH. As shown in Figure 4, the enhancement of dilution induced dissociation of bound ^{125}I-TSH by native hormone occurred in both specimens, irrespective of differences in equilibrium binding profiles. Thus, a lack of correlation between DEC and nonlinear Scatchard plots exists not only under nonphysiological conditions, in which binding is not of high affinity, but also when TSH is bound at physiological pH to a non-interacting, high-affinity component.

TABLE 1

Adenylate Cyclase Activity in Thyroid Membranes*.

TISSUE	BASAL	NaF	Gpp(NH)p	Gpp(NH)p + TSH
Normal	44±1.4	182±1.2	164±1.3	277±1.2
Adenoma	464±1.8	1046±1.1	1291±1.103	2108±1.2

* Production of cAMP was assayed in membranes (0.60 mg protein/ml) at 15 minutes, as desribed in "Methods". Final concentrations were: NaF (10 mM), Gpp(NH)p (1 μM), TSH (40 nM). Results are pmol cAMP $mg\ protein^{-1}\ min^{-1}$, expressed as means of triplicate determinations, ± standard deviation.

To verify the biological significance of TSH binding to the adenoma at pH 7.4, the adenylate cyclase responsiveness of these membranes was evaluated. Adenylate cyclase of both adenoma and normal tissue responded to TSH in the presence of the GTP analog, Gpp(NH)p (17), (Table 1), although basal activity of the adenoma was higher than that of normal, possibly reflecting increased cellularity. Adenylate cyclase dose-response curves for TSH were determined (Fig. 5), and expressed as a fraction of the maximal reaction velocity. It was seen that the relative response to TSH of the adenoma was equivalent to that of normal tissue, as determined by comparison of activation constants (10^{-7}M). This indicated that although qualitative differences in Scatchard profiles existed between the two tissues, both were equally functional with respect to adenylate cyclase responsiveness to TSH.[1]

Since it is generally accepted that the initial phase of TSH action entails activation of adenylate cyclase, these results illustrate the fact that DEC in

[1] Although discrepancies exist between the apparent Ka for adenylate cyclase and the K_d for TSH binding, it must be recognized that these assays are performed under different conditions as well as different concentrations of membrane protein. It has been established (5-7,17) that binding of TSH to thyroid membranes is significantly inhibited by $MgCl_2$, DTT, and nucleotides. Thus, quantitative comparisons cannot be made between TSH binding isotherms and adenylate cyclase dose response curves. Significantly, we have previously demonstrated (21) that in thyroid tumors lacking TSH binding, as determined under these assay conditions, TSH stimulation of adenylate cyclase is absent, although fluoride and Gpp(NH)p stimulated activity remains. This verifies that detection of specific TSH binding under these conditions represents a receptor coupled to adenylate cyclase.

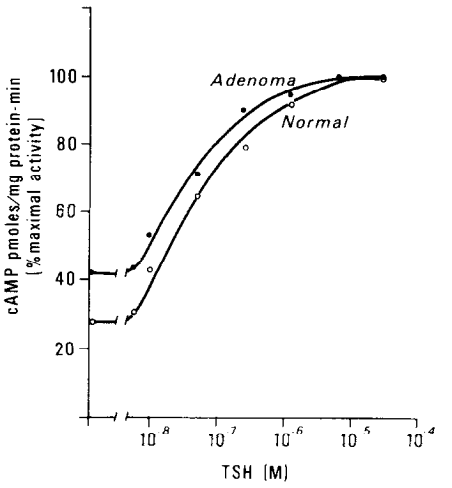

Fig. 5. Effect of varying concentrations of TSH on adenylate cyclase activity. Membranes (0.60 mg protein/ml) from adenoma (●) and adjacent normal thyroid (○) were assayed for cAMP production after 15 minutes, as described in "Methods". Results are the means of triplicate determinations, expressed as a percentage of maximal enzyme activity.

responsive thyroid membranes is clearly not related to the equilibrium binding profile, and therefore not valid proof of the negative cooperativity model. This is in agreement with previous studies on (-)-alprenolol binding to the β-adrenergic receptor of frog erythrocytes (18), and on lectin binding to human erythrocytes (19), where DEC was also observed in the absence of interactions between binding sites (linear Scatchard plot). Moreover, the presence of more than one class of TSH binding site, reflected by a non-linear Scatchard plot, is not essential in the hormonal modulation of the transmembrane signal *in vitro*. Evaluation of adenylate cyclase activity in this thyroid adenoma reveals normal responsiveness to TSH, even though only a single binding component is present. This suggests that there exist two distinct sites for binding of TSH in normal thyroid membranes, of which the high-affinity component is the biologically significant discriminator site, responsible for transduction of the hormonal message into the cell. Further support for this conclusion has come recently from studies of insulin binding to monocytes from patients with congenital lipodystrophy (20). It was shown that insulin resistance observed in these patients was correlated with the absence of high-affinity insulin receptor

while the low-affinity sites remained unchanged, supporting the heterogeneous non-cooperative nature of insulin binding.

While it appears that the high-affinity TSH binding site is solely responsible for adenylate cyclase stimulation, the role of the low-affinity site remains unclear. It is possible that the absence of this site may be responsible for the impaired function of the adenoma; similar observations have been made in malignant thyroid tumors, however, there appears to be no corresponding impairment of the adenylate cyclase response to TSH (21). Perhaps these changes in TSH binding are a general characteristic of diseased thyroid tissue, and reflect alterations in membrane composition which result in diminished low affinity binding. Further investigation into the intracellular events distal to the receptor-adenylate cyclase complex will enable us to locate the defect which renders these cells clinically unresponsive to TSH.

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