Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3460–3464, September 1974

The Contribution of Subunits of Thyroid Stimulating Hormone to the Binding and Biological Activity of Thyrotropin

(adenylate cyclase/luteinizing hormone/hormone receptors/membranes)

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Communicated by Terrell L. Hill, April 29, 1974

ABSTRACT **The binding of bovine TSH (thyroid stimulating hormone), LH (luteinizing hormone), and their subunits to the TSH receptor of beef thyroid membranes was compared to stimulation by these agents of adenylate cyclase [ATP pyrophosphate-lyase(cyclizing), EC 4.6.1.1] in the same membranes, glucose oxidation in dog thyroid slices, and the secretory process in mouse thyroids *in vitro* (colloid droplet formation) and *in vivo* (hormone release). The β-subunits of TSH and LH can bind to the TSH receptor and can activate thyroid function *in vitro*. In contrast, the α-subunit of TSH binds negligibly to the TSH receptor and has very low potency for stimulation of thyroid function (except for colloid droplet formation). Neither binding nor the biological activity of the β-subunits can be accounted for by TSH contamination, whereas this cannot be ruled out for α-TSH. LH binds to the TSH receptor even better than the β-subunit of TSH but the increased binding does not result in a corresponding activation of thyroid function. Neither α- nor β-TSH alone can induce more than 4–8% of the response to intact TSH in any of the investigated parameters. It is proposed that the β-subunit has within its structure the primary determinants which are necessary to stimulate biological activity, whereas the α-subunit imposes conformational changes on the β-subunit which in intact TSH promote binding *and* biological activity commensurately but in LH promote only binding.**

In a previous report we described the binding of [^{3}H]thyrotropin (TSH) to receptors on isolated bovine thyroid membranes. The specificity of [^{3}H]thyrotropin (TSH) binding to bovine thyroid membranes and its relationship to biological function were indicated (1). In the present study we have attempted to determine the contribution that the α- and β-subunits of TSH make to receptor binding and to biological effects measured *in vitro* and *in vivo*. The results have been compared with those obtained with LH (luteinizing hormone), which contains a nearly identical α-subunit but has a different β-subunit, and those obtained with β-LH.

METHODS

Assays. Binding and adenylate cyclase [ATP pyrophosphate-lyase(cyclizing), EC 4.6.1.1] assays were performed as previously described (1–3). Glucose oxidation was measured in dog thyroid slices (approximately 50 mg) by incubating them with 0.5 μCi of [*1*-^{14}C]glucose and collecting the $^{14}CO_2$ as described earlier (4). Colloid droplet formation was measured *in vitro* in mouse thyroids attached to the trachea (5). *In vivo* thyroid stimulating activity was measured by the method of McKenzie (6) and LH activity was measured by the method of Parlow (7). Protein was measured by the procedure of Lowry *et al.* (8). Membrane protein was determined after solubilization of appropriate aliquots by heating at 100° for 5–30 min in 1 N NaOH.

Preparation of Plasma Membranes. Beef thyroid plasma membranes were prepared by either of two procedures (1, 2). Although both preparations had the same [^{3}H]TSH binding properties, one preparation (2) had a 2- to 3-fold greater sensitivity to TSH stimulation of adenylate cyclase activity.

Hormone Preparations. Bovine TSH was purified as described (9, 10). An additional step, chromatography on Sephadex G-100, was added to eliminate residual contamina-

TABLE 1. *Bioassay activity of TSH and its subunits*

Hormone	Method of preparation	Thyroid stimulating activity, IU/mg	LH activity relative to NIH standard
TSH	Column chromatography*	24 ± 7	0.06
	Counter current†	27 ± 8	—
[^{3}H]TSH	Column chromatography*	21 ± 5	0.04
	Counter current†	—	—
α-TSH	Column chromatography*	0.3 ± 0.1	0.02
	Counter current†	0.4 ± 0.2	—
β-TSH	Column chromatography*	<0.025	<0.01
	Counter current†	<0.025	—
LH‡	—	<0.01	2.2–2.6
β-LH‡	—	<0.01	—

Thyroid stimulating activity was measured by a McKenzie bioassay (6), LH activity by the Parlow assay (7).

* Column chromatography, material purified by sequential chromatography on CM-cellulose, DEAE-cellulose, and Sephadex G-100 (9, 10). Subunits prepared from material purified by column chromatography (see *Methods*) were rechromatographed twice on Sephadex G-200 (Fig. 1).

† Counter current, material purified by DEAE-cellulose, counter current distribution, and Sephadex G-100. It was the same as preparations used to study the sequence of bovine TSH (13) and was a gift from Dr. John Pierce. Counter current subunits were also those prepared by Dr. Pierce (11, 14).

‡ A gift from Dr. John Pierce.

Abbreviations: TSH, thyroid stimulating hormone; LH, luteinizing hormone; ITP, inosine 5′-triphosphate.

tion of the TSH by its subunits or by the β-subunit of luteinizing hormone (11). [^{3}H]TSH was prepared using our modification (1, 9, 10) of the technique developed by Morell *et al.* (12). The [^{3}H]TSH was rechromatographed twice on Sephadex G-100 (11) to eliminate any residual nonprotein bound tritium, polymerization products, or subunits created by the reductive procedure. The specific radioactivity varied between 24.5 × 10^6 cpm/mg and 109.6 × 10^6 cpm/mg. Based on its unitage in the McKenzie assay, the [^{3}H]TSH gave a concentration-response curve with adenylate cyclase identical to unlabeled TSH.

TSH subunits were prepared by the method of Liao and Pierce (11). To insure complete elimination of TSH from these preparations, they were rechromatographed twice on Sephadex G-100 (Fig. 1). Their amino acid and carbohydrate compositions agreed with those previously reported (11).

The initial thyroid stimulating activities of our preparations of TSH- and TSH-subunits (purified by column chromatography) are summarized in Table 1 and are compared with analogous preparations (purified by counter current distribution) used for sequence studies of bovine TSH (13).

Bovine LH was a gift from Dr. John Pierce or was prepared as described (14, 15). Its LH activity was two to three times that of the NIH standard; its thyrotropic activity was less than 0.01 U/mg (Table 1). TSH preparations had negligible LH contamination when measured in bioassays (Table 1); they did not react with LH antisera in immunodiffusion analyses. The β-subunit of LH was prepared and had the carbohydrate and amino-acid compositions previously reported (13–17). Its TSH and LH activities are reported in Table 1.

RESULTS

Binding studies

As shown in Fig. 2*A*, [^{3}H]TSH binding to thyroid plasma membranes *in vitro* was decreased approximately 50% when an equimolar concentration of unlabeled TSH was included in the incubation mixtures. The α- and β-subunits of TSH also inhibited [^{3}H]TSH binding (Fig. 2*A*), and their inhibition was competitive with respect to TSH (Fig. 2*B*), i.e., binding of the subunits was to the TSH receptor. The relative affinities

TABLE 2. *[^{3}H]TSH binding to thyroid plasma membranes in the presence of unlabeled TSH, LH, or their subunits*

Unlabeled inhibitor	Concentration necessary to cause 50% inhibition of binding, nmoles/ml	Ratio to [^{3}H]TSH in the binding assay*
TSH	5.09	1.0
LH	41.7	8.3
β-TSH	203.6	40.7
α-TSH	~1,250†	~250
β-LH	356	~70

Binding assays were as in Fig. 2, i.e., they included 5 nmoles/ml of [^{3}H]TSH. The molecular weight values used to obtain these data were 27,500 for TSH and LH; 13,750 for α-TSH and β-TSH.

* Based on 5.09 nmoles/ml of unlabeled TSH required to displace one-half of the bound [^{3}H]TSH.

† Estimated from competition studies analogous to Fig. 2 but in which [^{3}H]TSH was 0.25 or 0.5 nmoles/ml.

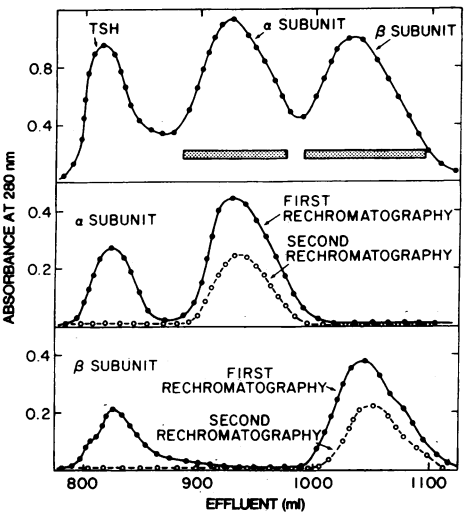

FIG. 1. *Upper panel.* Chromatography of bovine TSH (210 mg) after it was incubated with 1 M propionic acid in order to dissociate its subunits (11). The *stippled bars* denote the α-subunit and β-subunit peaks which were freeze-dried and subjected to rechromatography. *Middle panel.* Rechromatography of the α-subunit (63 mg) fraction (●) and of the α-subunit fraction obtained by this repeated chromatographic procedure itself (○). *Lower panel.* Rechromatography of the β-subunit (57 mg) fraction (●) and of the β-subunit fraction obtained by this repeated chromatographic procedure itself (○). All purifications were run on Sephadex G-100 columns (300 × 3 cm) at a flow rate of 24 ml/hr.

of α-TSH and β-TSH for the TSH receptors were calculated by comparing the concentrations which achieved 50% inhibition of [^{3}H]TSH binding (Table 2). LH also inhibited TSH binding by interacting with the TSH receptor (Fig. 2); its relative affinity to the TSH receptor as well as the relative affinity of β-LH, are compared to the affinities of the subunits of TSH in Table 2. With the exception of α-TSH, the

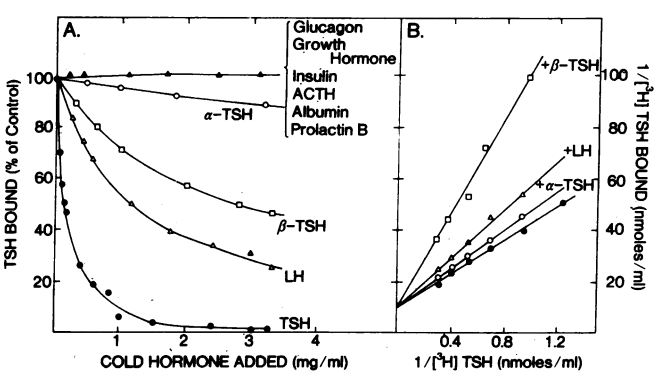

FIG. 2. (*A*) The effect of unlabeled hormones on [^{3}H]TSH binding to thyroid plasma membranes. Results are expressed as the percent of [^{3}H]TSH remaining bound when incubation mixtures contained increasing amounts of the unlabeled hormone during the entire incubation period. Assay conditions were standard (1) and included 1.0 mg of membrane protein. △, luteinizing hormone; □, β-subunit of TSH; and ○, α-subunit of TSH. [^{3}H]TSH was at a concentration of 5 nmoles/ml. (*B*) Reciprocal plot of TSH binding to thyroid plasma membranes as a function of increasing TSH concentration. Binding was performed with [^{3}H]TSH as the only hormone (●) and with ^{3}H[TSH] in the presence of 4 nmoles/ml of LH (△), 60 nmoles/ml β-TSH (□), or 145 nmoles/ml α-TSH (○).

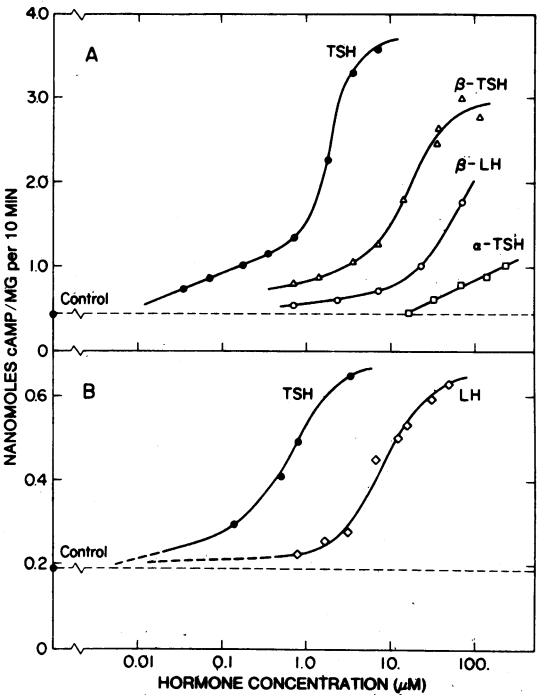

FIG. 3. The effect of TSH, LH, and their subunit on adenylate cyclase activity of beef thyroid membranes. All points are means of triplicates. In (*A*), all samples contained 5×10^{-5} M ITP. In (*B*), no ITP was present.

in vitro binding data (Table 2) differed significantly from the data for *in vivo* biological activity (Table 1). Both LH and β-TSH demonstrated much better *in vitro* binding activity than was suggested by their activities in the McKenzie bioassay. TSH contamination could not account for the discrepancy between *in vivo* and *in vitro* data.

Lissitzky *et al.* (18) have also reported the displacement of bound [^{131}I]TSH from TSH receptors by the subunits of TSH and by LH. In contrast to the results shown here, the molar ratios of subunits required to achieve 50% displacement in that study (18) can be estimated to differ by 2- to 25-fold despite similar levels of biological potency. Whether these discrepancies result from properties of the labeled hormone preparations or from the properties of the tissues used is not clear at present.

Biological activity studies

Adenylate Cyclase Activation. As can be seen in Fig. 3*A*, both TSH subunits were able to stimulate adenylate cyclase in beef thyroid plasma membranes; as with TSH (3), the subunits also stimulated in the absence of added ITP (not shown). The β-subunit of TSH exhibited approximately 6% of the molar activity of TSH, whereas the α-subunit possessed 0.5% of the molar activity of TSH. For the α-subunit, accurate comparison was not possible because the comparison had to be made on the flat component of the response curve. While these studies were in progress, Bloom and Field (19) described an effect of the α- and β-subunits of TSH (although purity of their materials was not ascertained). They concluded that the α- and β-subunits must have 2.5% or less of the receptor activity of TSH. As demonstrated here, the α- and β-subunits have approximately 0.4% and 2.5%, respectively, of the binding activity of TSH.

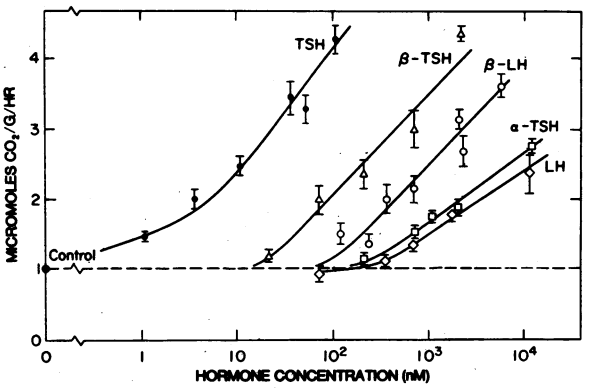

FIG. 4. Stimulation of glucose oxidation in dog thyroid slices by TSH, LH, and their subunits. Slices from thyroids of single dogs were preincubated at 37° for 30 min in Hank's balanced salt solution containing 0.1% bovine serum albumin and 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer pH 7.4, and 5.5 mM glucose. They were incubated for 60 min in fresh media containing, in addition, [*1*-^{14}C]glucose and hormone, where indicated. Values from different glands were normalized to the mean control of all experiments.

LH also stimulated adenylate cyclase activity in beef thyroid membranes and possessed about 5% of the molar activity of TSH in stimulating the cyclase of these membranes (Fig. 3*B*). It should be noted that the lower absolute activity of cyclase in Fig. 3*B* is the result of deleting ITP from the incubation mixture. Similar potency ratios have, however, been obtained for LH in the presence of ITP. When nonsaturating levels of LH were added to nonsaturating levels of TSH, the effects of adenylate cyclase stimulation were additive, i.e., LH did not inhibit the TSH stimulation.

The LH effect was surprising in that its subunit common with TSH, the α-subunit, had very low, if any, intrinsic activity, and in that its adenylate cyclase activity was significantly lower than its binding activity (Tables 1 and 2 and Figs. 2 and 3). β-LH had approximately half the adenylate cyclase activity of β-TSH and, surprisingly, this was about the same potency in this membrane system as exhibited by LH. In these studies, binding and adenylate cyclase stimulation by LH cannot be explained by TSH contamination. Physical and immunological studies alone should detect better than the 5% contamination necessary to obtain the binding results and these, plus the *in vivo* assay data (Table 1), eliminate TSH contamination as a cause for the adenylate cyclase effects.

The competitive nature of binding inhibition (Fig. 2) indicates that LH combines with the same receptors on the thyroid membranes as do TSH, α-TSH, and β-TSH. Therefore, at saturating TSH concentrations, LH might be expected to be a competitive inhibitor of TSH. Support for this possibility comes from experiments by Bloom and Field (19), whose studies on adenylate cyclase activation not only demonstrated no additive effect of TSH in the presence of *maximum* amounts of LH but, perhaps, a slight inhibition.

Glucose Oxidation. The stimulation of glucose oxidation in dog thyroid slices is a known, sensitive response to TSH. All of the present materials stimulated $^{14}CO_2$ formation from [*1*-^{14}C]glucose (Fig. 4). The potency of the subunits of TSH or LH in relation to native TSH was of the same order in this tissue as for cyclase stimulation of beef thyroid membranes.

On the other hand, LH was clearly *less* potent than its β-subunit. Whether this implies more stringent conformational requirements of the dog TSH receptor remains to be explored. It is again noted that the β-subunit exhibited approximately the same molar activity in this assay and in the binding assay (Figs. 2 and 3), whereas the LH activity was less than 0.01 its binding activity.

Colloid Droplet Formation. The effects of the TSH- and LH-subunits on colloid droplet formation are listed in Table 3. Submaximal concentrations of hormones and subunits were used to facilitate comparison. Under the same conditions a maximal response would be about 200 droplets/100 nuclei. All three subunits, β-TSH, α-TSH, and β-LH exhibited very similar potencies which amounted to 4–6% that of native TSH. The activity of LH was somewhat lower than that of the subunits. Although complete dose–response curves were not obtained, the surprisingly high activity of the α-subunit of TSH, and the similarity in potency of all subunits tested, suggest that the mouse system shows clearly different receptor discrimination than either beef or dog thyroid tissue. In view of the low *in vivo* activity of these subunits in the same species (Table 1, McKenzie assay), the metabolic fate of the subunits *in vivo* may play a large role in their low potency.

TABLE 3. *The colloid droplet response of mouse thyroids to TSH, LH, and their subunits*

Treatment	Concentration (nM)	Droplets per 100 nuclei ± SE
Control	—	1.4 ± 0.7
TSH	11	60.4 ± 6.7
β-TSH	179	56.9 ± 3.1
	18	8.6 ± 0.2
α-TSH	357	79.0 ± 9.0
	71	21.7 ± 0.8
β-LH	357	90.7 ± 12.1
	71	39.7 ± 14.2
LH	179	47.4 ± 17.2
	36	7.6 ± 1.5

Thyroids attached to the trachea were preincubated in Eagle's no. 2 medium containing 0.1% bovine serum albumin for 45 min. Glands were then removed and incubated for 60 min in 2 ml of fresh medium containing hormones as indicated. Subsequent treatment was performed as in (5).

DISCUSSION

Table 4 compares the activity of TSH to its α- and β-subunits and to LH in terms of binding, *in vitro* biological effects (adenylate cyclase activation, glucose oxidation), and biological effects in intact thyroids (colloid droplet formation, McKenzie bioassay). It is clear from these data that neither subunit alone can account for the binding activity or specificity of intact TSH, and the biological function of the subunits appears to reflect primarily the reduced ability to bind to the TSH receptor. β-TSH has at least a 6-fold greater binding activity than α-TSH, and its binding is associated with considerable *in vitro* biological activity. Comparison of the molar activity ratios for binding and *in vitro* biological activity suggests that β-TSH has the molecular determinants necessary to stimulate thyroid function but that it does not have all the required determinants present in intact TSH vital to binding. Its lower biological activity, therefore, mainly reflects its low binding activity. The very low *in vivo* activities of β-TSH show that TSH contamination cannot account for these data, and the discrepancy between *in vivo* and *in vitro* effects suggests that the β interaction with TSH receptors is diminished *in vivo*, perhaps as a consequence of extraneous factors such as a shorter circulating half-life, combination with an inactivating serum component, etc.

The low activity as measured by binding, stimulation of adenylate cyclase and glucose oxidation, as well as *in vivo* function, must be considered maximal activities of α-TSH. Although low absolute activity and the uniform molar activity ratios in most of these assays would be consistent with up to 0.4% TSH contamination (which cannot be excluded by either physical or immunological criteria), the "anomalously" high potency for droplet formation suggests that there must be some intrinsic activity in the α-subunit. The generally low activity of α-TSH thus reflects poor binding to the TSH receptor rather than an absence of determinants important to the transmission of the hormonal message to the cell.

It is known from subunit recombination studies (20–24) that the union of α-LH and β-TSH results in a molecule with the characteristics of TSH, whereas the union of α-TSH and β-LH results in an LH-like molecule. Since α-LH and α-TSH are nearly identical in primary structure (13, 16, 17), it is possible to view LH as the union of α-TSH with the "wrong" β-subunit, i.e., with an inactive structural analog of β-TSH. Given such a view, it is notable that LH binds better than either subunit alone, yet it causes an inversion of the ratio of

TABLE 4. *The relative activity of TSH, LH, and their subunits on TSH binding and on thyroid function (% molar activity)*

Hormonal factor	Binding activity* (beef)	Adenylate cyclase activation† (beef)	Glucose oxidation ‡ (dog)	Colloid droplet formation§ (mouse)	Thyroid hormone release (McKenzie bioassay)¶ (mouse)
TSH	100	100	100	100	100
α-TSH	0.4	~0.1	0.2	4	0.5
β-TSH	2.5	8	5	5–6	<0.05
LH	12.5	5	0.1	2.5	<0.05
β-LH	1.5	2	1	5	<0.05

Percent molar activity defines the molar activity of the subunit or LH preparations relative to TSH when the molar activity of TSH is set at 100. % molar activity = (moles subunit/moles TSH) × 100.

* Data derived from Fig. 2 and Table 2. † Data derived from Fig. 3. ‡ Data derived from Fig. 4. § Data derived from Table 3. ¶ Data derived from Table 1.

binding activity to adenylate cyclase activation, from approximately 1 to 3 (β-TSH alone) to approximately 2.5 to 1 (LH). The relatively low binding ability of β-LH and its relatively high activity for adenylate cyclase suggest that, unlike TSH, upon association of β-LH with its α-subunit in native LH, no enhancement of message propagation in the membrane results despite improved binding. These data, therefore, suggest that the α interaction with the β-subunit induces structural changes which increase the ability to bind to the TSH receptor. However, this change in the structure of the β-subunit adversely affects determinants required to activate thyroid function, or else adenylate cyclase activation would increase proportional to binding. In contrast, dimer formation of the α-subunit with β-TSH produces no such adverse (conformational) effects and binding and thyroid stimulation are enhanced *pari passu*.

In sum, these data allow us to speculate that the β-subunit is vital to the expression of the biological activity of TSH; that the α-subunit is critical to binding and specificity not because of any direct α activity but because of the conformational information it provides to the β-subunit in the α–β union; that specificity is a function of the molecular conformation resulting from the α–β union rather than from the structure of either subunit alone; and that there is a critical orientation of the β-subunit within the receptor which is necessary for biological activity.

Rayford *et al.* (25) have used specific antisera to demonstrate that the biological activity of some β-human chorionic gonadotropin preparations reflects contamination by small amounts ($<2\%$) of native human chorionic gonadotropin. Analogous experiments with bovine TSH and its subunits should be performed. However, the absence of specific antisera has thus far restricted such an evaluation. In the present report we have used a repetitive purification technique (Fig. 1) in order to minimize contamination and the device of multiple parallel assays of biological effects to measure the limits of native hormone contamination. This last device is recommended as a reasonably sensitive index of subunit contamination by native hormones.

We are indebted to Dr. John Pierce, Department of Biochemistry, University of California Center for Health Sciences, Los Angeles, Calif., who provided us with LH, TSH, α-TSH, and β-TSH samples for use as standards against which we could rigorously evaluate our TSH and subunit preparations.

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