Experimental Exophthalmos

BINDING OF THYROTROPIN AND AN EXOPHTHALMOGENIC FACTOR DERIVED FROM THYROTROPIN TO RETRO ORBITAL TISSUE PLASMA MEMBRANES

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Biologically active preparations of ¹²⁵I-thyrotropin, [³H]thyrotropin, and the [³H]exophthalmogenic factor derived from thyrotropin by partial pepsin digestion have been used to study the binding properties of the thyrotropin receptor on guinea pig retro-orbital tissue plasma membranes. In regard to the optimal conditions of binding, pH, buffer, salt concentrations, and temperature, these properties are the same as those described in any accompanying report concerning thyrotropin binding to bovine thyroid plasma membranes (Tate, R. L., Schwartz, H. I., Holmes, J. M., Kohn, L. D., and Winand, R. J. (1975) J. Biol. Chem. 250, 6509–6515). In addition, thyrotropin receptors on the retro-orbital tissue plasma membranes are similar to thyrotropin receptors on bovine thyroid plasma membranes in their apparent negative cooperativity and in their relative affinities for luteinizing hormone, the β subunit of thyrotropin, and the α subunit of thyrotropin. In contrast, γ-globulin from patients with malignant exophthalmos enhances binding when added to incubation mixtures containing the retro orbital tissue plasma membranes but not when added to those containing thyroid plasma membranes. Normal γ-globulin and γ-globulin from Graves' disease patients without exophthalmos do not have this property. The γ-globulin itself does not bind to the membrane except in the presence of thyrotropin or its exophthalmogenic factor derivative.

Tryptic digestion of the retro-orbital tissue membranes releases specific thyrotropin and exophthalmogenic factor binding activity into the supernatant phase. Chromatography on Sephadex G-100 indicates that this trypsin-released receptor activity has a molecular weight of 75,000 or greater, rather than 15,000 to 30,000 for the trypsin-released receptor activity from bovine thyroid membranes (Tate, R. L., Schwartz, H. I., Holmes, J. M., Kohn, L. D., and Winand, R. J. (1975) J. Biol. Chem. 250, 6509–6515).

As a consequence of our studies on experimental exophthalmos (1–9), we have suggested that human exophthalmos is a two-factor disease caused by the simultaneous presence of thyrotropin or a derivative of the TSH⁺ molecule with no thyroid-stimulating ability and by the presence of an abnormal or autoimmune γ-globulin. The TSH or TSH derivative served as the direct effector while the γ-globulin targeted this effector by increasing its binding to retro-orbital tissue. The evidence that led to this hypothesis can be summarized as follows: (a) the thyrotropin molecule is exophthalmogenic (3); (b) the thyrotropin molecule can be fragmented by partial pepsin digestion to yield a derivative (EPF) with exophthalmogenic activity but no thyroid-stimulating ability (4, 5); (c) this derivative as well as TSH induces exophthalmos and the biochemical changes characteristic of exophthalmos in the guinea pig, a mammalian model of exophthalmos (6); (d) there is an exophthalmogenic factor in the sera of exophthalmic patients which is in the γ-globulin fraction of these sera and is not the long-acting thyroid stimulator (LATS) (1, 7); and (e) this γ-globulin from the sera of exophthalmic patients significantly increases the in vitro binding of TSH and the TSH-derived exophthalmogenic factor to plasma membranes of guinea pig retro-orbital tissues (Harderian glands), but not thyroid plasma membranes (8, 9).

The preliminary binding studies which resulted in the last finding (8) were performed using thyrotropin and the exoph-
thalmogenic derivative of the TSH molecule which had been labeled with tritium. In the present study we have further characterized the properties of the TSH receptor in retro-orbital tissues, not only with [3H]TSH and [3H]EPF, but also with a biologically active [123I]TSH preparation (10) which is used at hormone concentrations in vitro which could be expected in vivo. The data show that the binding properties of the TSH receptors of retro-orbital tissue plasma membranes are effectively the same as those described for TSH receptors on bovine thyroid plasma membranes (9, 10) with the exception of the γ-globulin effect previously reported (8) and further characterized herein. Most important in this report, tryptic digestion is shown to release the TSH and EPF binding activity of the retro-orbital tissue membranes into the supernatant phase as a component which has a significantly higher molecular weight than the fragment released by tryptic digestion of thyroid plasma membranes (10).

### Materials and Methods

**Hormone and γ-Globulin Preparations**—Purified bovine TSH, [3H]TSH, and [3H]EPF were purified as previously described (3-6, 8-10). The properties of these preparations are detailed in the accompanying report (10). The unlabeled and γ-labeled exophthalmogenic factor ([3H]EPF) preparations were prepared from purified TSH and purified [3H]TSH preparations by partial pepsin digestion and electrofocusing as described (4). The exophthalmogenic activities (11, 12) of the purified TSH, [3H]TSH, and [3H]EPF preparations were 21 ± 6, 19 ± 4, and 16 to 20 units/mg, respectively. Units are defined by the exophthalmogenic activity of a crude commercial preparation of TSH (Amihon, Oss, Holland) and are recorded as equivalent TSH units of that preparation (11, 12). The thyroid-stimulating activities (13) of the unlabeled and [3H]EPF preparations were less than 0.1 unit/mg; the specific radioactivities of the [3H]EPF preparations were between 0.19 and 0.24 Ci/mm.

Unlabeled TSH subunits and luteinizing hormone were prepared as described (14-17) as were preparations of γ-globulin from normal sera, from the sera of patients with Graves' disease and malignant exophalmonia (2, 10, 18). As noted before (8-10), γ-globulin preparations were exhaustively dialyzed against 0.01 M ammonium bicarbonate and lyophilized before being evaluated for LATS activity, exophthalmogenic activity, or activity in binding assays. [123I]γ-globulin preparations were prepared by either of the two methods (19, 20) used to label the unlabeled hormone preparations or by partial pepsin digestion of their biological activity after iodination was determined to be unimpaired by criteria of either fish bioassays (11, 12), LATS bioassay (21), or receptor binding assays (8-10).

**Preparation of Plasma Membranes**—Plasma membranes of retro-orbital tissue were prepared from Hordfern glands of guinea pigs followed by modification of our previous procedure (8) which used the sucrose density gradient centrifugation technique of Neville (22). The modification included a 10-fold increase in both the tissue homogenized and in the volume of homogenizing buffer, initial centrifugations at 7500 x g rather than 3000 x g, and suspension of the crude membrane pellet in 40 ml rather than 1.5 ml of 20 mM Tris-HCl (pH 7.5) containing 1 mM ethylenediaminetetraacetate. The discontinuous sucrose gradient centrifugation (8, 22) was in a Beckman model No. L5-65 ultracentrifuge and was preceded by centrifugation at 25,000 rpm. Plasma membranes were harvested from the 30 to 40% interface, were diluted 2- to 3-fold with water, and were centrifuged at 25,000 rpm in the same rotor. The final pellet was suspended as a particulate suspension in 0.01 M NH4HCO3, pH 7.3, and was stored in 1- to 3-ml aliquots in liquid nitrogen. Individual tubes were thawed and appropriately diluted before use.

Membranes prepared in this manner appeared to be large vesicles with a triple lamellar structure at high magnification and appeared to be minimally contaminated by other cellular components (8). They demonstrated an increase in specific activity of membrane associated enzymes (5'-nucleotidase, 19-fold; phosphoenolpyruvate carboxykinase, 24-fold; acid phosphatase, 16-fold; succinate dehydrogenase, 16-fold; TPNH-cytochrome c reductase, 14-fold; and pyruvate carboxylase, 17-fold); these values are improved over those previously reported (8).

Adenylyl cyclase activity was measured by following the procedure of Krishna et al. (23) as modified by Wolff and Jones (24) and used the optimal conditions described (25). Standard methods were used for the assay of 5'-nucleotidase (26), pyruvate carboxylase (27), phosphoenolpyruvate carboxykinase (28), TPNH cytochrome c reductase (29), succinate dehydrogenase (30), magnesium-activated ATPase (31), and acid phosphatase (32).

### Results

**Binding Properties of Retro-orbital Tissue Plasma Membranes**—The binding of [125I]TSH, [3H]TSH, and [3H]EPF to guinea pig retro-orbital tissue plasma membranes had the same pH optimum and the same pH dependency as detailed in an accompanying report (10) for the binding of [125I]TSH or [3H]TSH to bovine thyroid plasma membranes. In a like manner there was a similar sensitivity to the choice and concentration of buffers either by comparison among each of the labeled preparations or by comparison to the TSH receptors of thyroid membranes (10). Optimal binding was thus in 0.025 M Tris-acetate or Tris-maleate independent of the labeled hormone preparation and independent of the TSH receptors being studied, bovine thyroid or guinea pig retro-orbital.

The binding of all three hormone preparations was sensitive to the concentration of salts in the incubation medium and was especially sensitive if these salts included magnesium or calcium ions. These data are again the same as those obtained in our accompanying studies of [125I]TSH and [3H]TSH binding to bovine thyroid tissues (10).

**Binding of [3H]TSH and [3H]EPF** was both immediate and maximal at either 0, 25, or 37° (Fig. 1). Continued incubation at 0° resulted in no decrease in [3H]TSH or [3H]EPF binding, whereas continued incubation at 25 and 37° resulted in a progressive decrease in binding activity. [125I]TSH binding was effectively immediate at 37°; at 25° it took 5 min to reach the maximal level; and at 0° it took nearly 30 min (Fig. 1). Again, continued incubation at 0° had no effect, whereas continued incubation at 25 or 37° caused progressive decreases in binding. Changes in the [125I]TSH concentration correlated inversely with changes in the time of binding; thus, a 10-fold decrease in concentration prolonged the time to reach maximal binding to almost 2 hours at 0° and a 10-fold increase in concentration decreased the time optimal binding to less than 1 min at 0°.

These data are once again analogous to those described for [125I]TSH and [3H]TSH binding to thyroid plasma membranes in the accompanying report (10). Like the thyroid membrane-TSH system, the temperature sensitivity appears to be a problem of membrane stability (Table I) rather than enzymatic destruction of the hormone. Also like the thyroid membrane-TSH system, the hormone receptor interaction exhibits time dependency when the concentration of one of its reactants is varied, a phenomenon to be predicted in a bimolecular binding reaction.
The binding of each of the hormonal preparations, \([\text{TSH}]\) and \([\text{EPF}]\), was optimal except for binding being performed at the noted time after incubation was initiated. In (A) \([\text{Y-TSH}]\) was at \(5 \times 10^{-11} \text{ M}\) in (A). In (B) \([\text{Y-TSH}]\) and \([\text{EPF}]\) were at \(5 \times 10^{-7} \text{ M}\).

In the presence of a constant amount of membrane protein and at concentrations of \([\text{TSH}]\) and \([\text{EPF}]\) greater than \(5 \times 10^{-7} \text{ M}\), saturation kinetics prevailed since 6% or less of the hormone added to the incubation media was bound. Under these conditions unlabeled TSH inhibits \([\text{TSH}]\) binding in a clearly theoretical fashion, i.e. the addition of equal concentrations of labeled and of unlabeled hormone to an incubation mixture resulted in approximately a 50% inhibition of binding. To achieve the same level of inhibition required 4 to 5 times higher concentrations of the unlabeled exophthalmogenic derivative of the TSH molecule \((\text{EPF})\) isolated from partial pepsin digest of purified TSH preparations. Double reciprocal plots of the \([\text{TSH}]\) binding data in the range of \(5 \times 10^{-7} \text{ to } 5 \times 10^{-6} \text{ M}\) \([\text{TSH}]\) indicated that the exophthalmogenic factor was a purely competitive inhibitor of \([\text{TSH}]\) binding.

These data thus indicated that TSH and the exophthalmogenic derivative of the TSH molecule were binding at the same receptor site and that the affinity of the TSH for the receptor was approximately 4 to 5 times greater than the affinity of the exophthalmogenic factor to this receptor. In contrast, the affinity of TSH to TSH was nearly 10 times greater than the affinity of the exophthalmogenic factor to this receptor. In contrast, the affinity of TSH to the receptor was nearly 10 times greater than the affinity of the exophthalmogenic factor to this receptor.
binding as a function of hormone concentration revealed the fact that there was significant nonlinearity in the Scatchard plots. One possible explanation for this nonlinearity was the existence of independent groups of TSH receptors with different binding constants and, in fact, two such groups had been visually segregated, one of high affinity \( (K = 0.25 \times 10^{-10} \text{ M}^{-1}) \) and one of low affinity \( (K = 0.25 \times 10^{-10} \text{ M}^{-1}) \). A more likely explanation was suggested by Hill plots of these data which indicated that these were not behaving independently but were exhibiting a negatively cooperative relationship since the slope of such plots was less than unity. It was therefore not surprising that \(^{131}I\)-TSH binding to retro-orbital tissue plasma membranes resulted in a similarly nonlinear Scatchard plot \((34, 35)\) and that, although this plot could be interpreted to reflect the existence of a group of high affinity \( (K = 0.12 \times 10^{-13} \text{ M}^{-1}) \) and low affinity \( (K = 0.08 \times 10^{-9} \text{ M}^{-1}) \) TSH receptors (Fig. 2), the Hill plot \((36, 37)\) of these data (not shown) had a slope of 0.7. Thus, like the TSH receptors on bovine thyroid plasma membranes, all of the retro-orbital TSH receptors had the same pH optima, buffer optima, salt inhibition phenomena, and temperature effects independent of their affinity for TSH, and the retro-orbital tissue TSH receptors appeared to be related in a negatively cooperative sense \((38, 39)\).

The specificity of \([\text{H}]\)TSH and \([\text{H}]\)EPF binding to retro-orbital plasma membranes was indicated since albumin, adrenocorticotropin hormone, glucagon, insulin, and human growth hormone had no effect on binding and since muscle membranes were shown in a previous report to have negligible binding of either tritiated derivative. Analogous specificity data have been obtained using \(^{125}I\)-TSH as the measure of TSH receptor in retro-orbital tissue plasma membranes. In experiments analogous to those described in Table III of our accompanying report \((10)\) on thyrotropin binding to thyroid plasma membranes, we have demonstrated that unlabeled TSH or EPF could not only compete for the binding of \(^{125}I\)-TSH but could also reverse or chase it from the retro-orbital tissue plasma membranes.

**Effect of \(\gamma\)-Globulin on TSH Binding to Retro-orbital Tissue Receptors and Effect of TSH or Its Exophthalmogenic Derivative on \(\gamma\)-Globulin Binding**—In our preliminary report, we demonstrated that the binding of \([\text{H}]\)TSH and \([\text{H}]\)EPF was enhanced by the addition of the incubation medium of \(\gamma\)-globulin from patients with malignant exophthalmos whereas binding was not enhanced by either normal \(\gamma\)-globulin or \(\gamma\)-globulin from patients with Graves' disease but no exophthalmos \((8)\). Studies of the effect of the \(\gamma\)-globulin from malignant exophthalmos on the binding of \(^{125}I\)-TSH and \([\text{H}]\)TSH to the retro-orbital membrane receptors were performed in order to evaluate the effect of the abnormal globulin even at \(^{125}I\)-TSH concentrations in the \(10^{-8} \text{ M} \) to \(10^{-9} \text{ M} \) range (Fig. 2). From the effect on the Scatchard plot (Fig. 2, inset), and from the finding that the Hill coefficient is shifted from 0.7 to 0.87 it appears that the presence of the \(\gamma\)-globulin from malignant exophthalmic patients results in an increase in the number of sites which can recognize the hormone, in an increase in their affinity to the hormone, and in an alteration of the negatively cooperative relationship between the sites.

The role of the \(\gamma\)-globulin in this process was investigated using \(^{125}I\)-\(\gamma\)-globulin as the binding agent rather than labeled hormone (Table II). Alone, the \(^{125}I\)-\(\gamma\)-globulin from patients with malignant exophthalmos did not bind better than either \(\gamma\)-globulin from normals or from Graves' disease patients without exophthalmos and whatever did bind could be prevented by the addition of unlabeled normal \(\gamma\)-globulin to the incubation mixture (Table II). In contrast, the addition of either unlabeled TSH or its unlabeled exophthalmogenic derivative to the incubation mixture resulted in significant \(^{125}I\)-\(\gamma\)-globulin binding in incubations containing the malignant exophthalmogenic \(\gamma\)-globulin preparations but not in incubations containing the other \(\gamma\)-globulin preparations. Normal \(\gamma\)-globulin and \(\gamma\)-globulin from Graves' disease patients without exophthalmos could not significantly inhibit this binding, whereas unlabeled \(\gamma\)-globulin from either the same or different patient with malignant exophthalmos was able to compete.

**Effect of Trypsin on TSH Receptor from Guinea Pig Retro-orbital Tissue Membrane**—In our accompanying report \((10)\), we showed that trypsinization destroyed the TSH binding activity on bovine thyroid plasma membranes and that trypsinization released into the supernatant a 15,000 to 30,000 molecular weight receptor fragment which had specific binding activity. Analogous trypsinization of retro-orbital tissue plasma membranes also destroys their TSH and exophthalmogenic derivative binding activities and releases specific binding activity into the media (Fig. 3). In contrast to the thyroid receptor released, however, the receptor activity released by trypsinization of retro-orbital tissue membranes

### Table II

**Binding of \(^{125}I\)-labeled \(\gamma\)-globulin to retro-orbital tissue membranes**

<table>
<thead>
<tr>
<th>Unlabeled additions to incubation containing (^{125}I)-(\gamma)-globulin</th>
<th>(^{125}I)-(\gamma)-globulin from a patient with malignant exophthalmos*</th>
<th>(^{125}I)-(\gamma)-globulin from a LATS positive patient with Graves' disease but no exophthalmos*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>890</td>
<td>920</td>
</tr>
<tr>
<td>+ exophthalmogenic (\gamma)-globulin, 30 (\mu)g</td>
<td>450</td>
<td>530</td>
</tr>
<tr>
<td>+ normal (\gamma)-globulin, 30 (\mu)g</td>
<td>480</td>
<td>515</td>
</tr>
<tr>
<td>+ TSH*</td>
<td>12,400</td>
<td>840</td>
</tr>
<tr>
<td>+ EPF*</td>
<td>15,200</td>
<td>950</td>
</tr>
<tr>
<td>+ TSH and exophthalmogenic (\gamma)-globulin, 30 (\mu)g</td>
<td>6,800</td>
<td>490</td>
</tr>
<tr>
<td>+ EPF and exophthalmogenic (\gamma)-globulin, 30 (\mu)g</td>
<td>7,400</td>
<td>520</td>
</tr>
<tr>
<td>+ TSH and normal (\gamma)-globulin, 30 (\mu)g</td>
<td>11,600</td>
<td>590</td>
</tr>
<tr>
<td>+ EPF and normal (\gamma)-globulin, 30 (\mu)g</td>
<td>14,500</td>
<td>510</td>
</tr>
</tbody>
</table>

*The \(^{125}I\)-labeled \(\gamma\)-globulin from a malignant exophthalmos patient was biologically active in the fish bioassay. It was incubated with retro-orbital tissue plasma membranes using the same optimal conditions as described for TSH binding assays with the exception that no labeled TSH was included in the incubation. There were 30 \(\mu\)g of the labeled \(\gamma\)-globulin present in the 100 \(\mu\)l incubation mixture, i.e., it was twice the concentration in Fig. 2 and was at its maximal point of stimulation. The \(^{125}I\)-labeled \(\gamma\)-globulin had 230,000 cpm/30 \(\mu\)g.

*The \(^{125}I\)-labeled \(\gamma\)-globulin from a LATS positive Graves' disease patient without exophthalmos was prepared as described and was biologically active in a LATS assay \((21)\). It was incubated in the same way and at the same concentration as the malignant exophthalmos \(\gamma\)-globulin; 30 \(\mu\)g had 290,000 cpm. Analagous data to those presented in this column were obtained if \(^{125}I\) normal \(\gamma\)-globulin was used at the same concentration and similar specific activity.

* TSH was added at \(5 \times 10^{-7} \text{ M} \).
* EPF was added at \(5 \times 10^{-10} \text{ M} \).
eluted at the front of a G-100 column (Fig. 4) (molecular weight > 75,000) and on sucrose gradients (40) had a molecular weight of between 100,000 and 150,000. The solubilized receptor activity was specific for [\(^{3}H\)TSH, [\(^{3}H\)]TSH, and [\(^{3}H\)]EPF eluted at the front of a G-100 column (Fig. 4) (molecular weight ≈ 65,000) and on sucrose gradients (40) had a molecular weight > 75,000) and on sucrose gradients (40) had a molecular weight of 750,000. The solubilized receptor fragments described in an accompanying report (41). Controls included duplicate incubations and assays containing trypsin or trypsin plus trypsin inhibitor at identical concentrations but either in the absence of membranes added only at the time of the binding assay.

**Fig. 3.** Loss of binding activity in retro-orbital tissue plasma membranes (○), □, △ after exposure to trypsin and the release of receptor activity (O, □, △) into the supernatant solution. Membranes were exposed to 1-1-losamido-2-phenylethyl chloromethyl ketone trypsin at room temperature, at a 20:1 weight ratio of membranes to trypsin and in 0.025 M Tris-maleate, pH 7.8. At the noted times, aliquots were removed and mixed with an amount of cold soybean trypsin inhibitor (Calbiochem) which was 5-fold in excess of the trypsin concentration, and the suspensions were chilled to 2-4°C. Aliquots from this suspension were immediately assayed for (A) binding activity in the presence of [\(^{125}I\)]TSH (○) and (B) binding activity in the presence of [\(^{3}H\)]TSH (□) or [\(^{3}H\)]EPF (△). Other aliquots were centrifuged in a Beckman microfuge and the supernatants were assayed (A) for [\(^{125}I\)]TSH binding activity (○) and (B) for [\(^{3}H\)]TSH (□) and [\(^{3}H\)]EPF (△) binding activity using a modified binding assay developed in our studies on solubilized TSH receptors (41). Controls included duplicate incubations and assays containing trypsin or trypsin plus trypsin inhibitor at identical concentrations but either in the absence of membranes added only at the time of the binding assay.

**Fig. 4.** Chromatography on Sephadex G-100 of the TSH receptor activity released into the supernatant and trypsinization of retro-orbital tissue plasma membranes. Binding activity was measured under optimal conditions for the solubilized receptor assay (3) and using both [\(^{125}I\)]TSH (○) and [\(^{3}H\)]EPF (△) at 5 × 10\(^{-10}\) M and 5 × 10\(^{-7}\) M concentrations. Solubilized receptor activity was prepared as described in Fig. 3; the supernatant after 20 min of a large scale incubation was used. The column was 50 × 0.9 cm in size and was equilibrated with 0.025 M Tris-acetate, pH 7.0. The elution rate was 20 ml/hour. Fractions of 0.1 ml were collected. The void volume is the peak of elution of a dextran blue dye marker > 200,000 in molecular weight; albumin (67,000), TSH (27,000 to 28,000), and β-TSH (13,000–14,000) were markers used to calibrate the column. The elution area of the trypsin solubilized TSH receptor fragment described in an accompanying report (10) is noted by the dashed line. The same data were obtained if the column was eluted in 0.001 M bicarbonate, pH 9.5, containing 0.1% Triton X-100.

**REFERENCES**


**DISCUSSION**

The present report shows that in terms of its binding properties and with the exception of the γ-globulin effect on binding, the TSH receptor on retro-orbital tissue plasma membranes has many similarities to the TSH receptor on bovine thyroid plasma membranes. The data indicate that the γ-globulin from malignant exophthalmos patients enhances TSH binding or the binding of its exophthalmogenic derivative to retro-orbital tissue plasma membranes by increasing the number of TSH receptors, by shifting the association constants of receptors to much lower levels, and by decreasing the negatively cooperative relationship among the receptor sites. Most important in this regard, the data show that the γ-globulin does not modify the binding site or the receptor directly. Its action requires the presence of TSH or its exophthalmogenic derivative; hence, the γ-globulin must function either to modify the TSH or EPF molecule itself or to interact with the membrane only after TSH has been bound. Independent of the mechanism by which the γ-globulin acts, a different receptor site topography must be presupposed in retro-orbital tissue membranes as opposed to thyroid membranes in order to account for the different functional response to the γ-globulin. In this regard, trypptic release of different sized receptor fragments from retro orbital and thyroid plasma membranes is highly suggestive. The strong conservation of pH, salt, temperature, and kinetic properties between the receptors argues for the relationship of this structural difference to the γ-globulin effect, although the different animal species from whence the receptor came must be considered. Final proof will require further experimental efforts.

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