A water-soluble fragment of the thyroid-stimulating hormone receptor which binds both thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies

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ABSTRACT

Previous studies have shown that freezing and thawing of human thyroid homogenates releases a water-soluble substance which reversibly binds to TSH-receptor antibodies. This substance has been designated long-acting thyroid stimulator absorbing activity (LAA). We now describe a new method for measuring LAA based on the TSH-receptor assay and application of the technique to the study of LAA.

Our results indicate that LAA is a heat-labile glycoprotein which co-elutes with haemoglobin on gel filtration. Furthermore, LAA is retarded by columns of Sepharose–TSH but not by Sepharose coupled to human chorionic gonadotrophin, normal immunoglobulin G or bovine serum albumin, suggesting that LAA contains a binding site for TSH as well as for TSH-receptor antibodies. It would seem therefore that LAA is a water-soluble fragment of the TSH receptor possibly resulting from proteolytic cleavage of the receptor at a site close to the cell surface.


INTRODUCTION

The sera from patients with hyperthyroid Graves’ disease contain antibodies to the thyroid-stimulating hormone (TSH) receptor which can act as TSH agonists (Rees Smith, 1981). This type of antibody was originally referred to as long-acting thyroid stimulator (LATS) because of its prolonged time-course of action in the bioassay system used for its measurement (Dorrington & Munro, 1966).

Frozen and thawed homogenates of human thyroid membranes contain a water-soluble substance which reversibly inactivates LATS (Smith, 1970, 1971; Dirmikis & Munro, 1973). This substance has been referred to as LATS absorbing activity (LAA) and as it binds TSH-receptor antibodies it would seem likely that LAA is closely related to the TSH receptor. We now describe an investigation of this possibility using a new method for measuring LAA based on a TSH-receptor antibody assay (Petersen, Dawes, Rees Smith & Hall, 1977; Kotulla & Schlesener, 1981; Shewring & Rees Smith, 1982).

MATERIALS AND METHODS

Serum samples

Normal pool serum was obtained by mixing sera from five or more healthy volunteers. Sera from patients with Graves’ disease were selected for study on the basis of high levels of TSH-receptor antibody as measured by the TSH-receptor antibody assay (Shewring & Rees Smith, 1982). All samples were stored in aliquots at –70°C and repeat freezing and thawing was avoided.

Preparation of detergent-solubilized TSH receptors, normal immunoglobulins and TSH

Detergent-solubilized TSH receptors were prepared from porcine thyroids (obtained from the local abattoir) as described previously (Rees Smith & Hall, 1981; Rickards, Buckland, Rees Smith & Hall, 1981). Immunoglobulins were prepared from normal human serum by ammonium sulphate precipitation (Hammarsten, 1878; Mehu, 1878; Edsall, 1947; Rees Smith...
& Hall, 1981). Thyroid-stimulating hormone (60–70 units/mg) was prepared from bovine pituitary glands (obtained from the local abattoir) by a method based on the purification procedure of Pierce, Liao, Howard et al. (1971) and labelled with \(^{125}\)I (Amer-sham International plc, Bucks) using the Iodogen method (Fraker & Speck, 1978) and receptor purified (Rees Smith & Hall, 1981). Iodogen was purchased from Boehringer Corporation Ltd, Lewes, Sussex.

**Preparation of LAA**

Human thyroid tissue was obtained at partial thyroidectomy for Graves’ disease, cut into segments (approximately 200 mg), quick-frozen in \(n\)-hexane at \(-70^\circ\)C and stored at \(-70^\circ\)C. When required, the tissue was allowed to thaw at room temperature, cut finely with scissors and a scalpel and homogenized (Polytron homogenizer) in 2 volumes of ice-cold buffer (50\(\mu\)M-NaCl, 10\(\mu\)M-Tris-\(\text{HCl}\), pH 7.5 and 3\(\mu\)M-Na\(\text{NaN}_3\)) (Tris/NaCl). The homogenate was filtered through muslin twice, allowed to freeze at \(-20^\circ\)C and then stored at \(-70^\circ\)C. Crude LAA was obtained by allowing the thyroid extract to thaw out slowly and then centrifuging at 100000 \(g\) for 1 h at 4 \(^\circ\)C. The supernatant fraction was immediately placed on ice and used within a few hours. Water-soluble extracts of human placenta were prepared in the same way as LAA for control studies.

In some experiments a crude membrane preparation was sedimented (20000 \(g\); 20 min; 4 \(^\circ\)C) from the thyroid homogenate before freezing at \(-20^\circ\)C. The sediment was suspended in Tris/NaCl (1 ml/g starting thyroid tissue), placed on ice and used within a few hours.

**Assay of LAA**

**Receptor assay of LAA**

This procedure employed a modification of the TSH-receptor antibody assay described previously (She-wring & Rees Smith, 1982). Samples (0–500 \(\mu\)l) of the solution to be tested for LAA were incubated with 50 \(\mu\)l normal pool sera for 1 h at 37 \(^\circ\)C while identical series of samples were incubated simultaneously with 50 \(\mu\)l Graves’ serum. Each reaction volume was adjusted to 550 \(\mu\)l by addition of Tris/NaCl containing 1 mg bovine serum albumin/ml (Tris/NaCl/BSA). Triplicate 100 \(\mu\)l samples were incubated with 50 \(\mu\)l detergent-solubilized porcine TSH receptors for 15 min at room temperature and then for 1 h at 37 \(^\circ\)C in the presence of 100 \(\mu\)l \(^{125}\)I-labelled TSH (5000 c.p.m.). The reaction mixture was made up to a volume of 500 \(\mu\)l with Tris/NaCl/BSA and 500 \(\mu\)l 30\% (w/v) polyethylene glycol (mol. wt 4000) in 1 \(m\)-NaCl were added. After mixing well, the tubes were centrifuged (1500 \(g\); 45 min; 4 \(^\circ\)C) and the pellet was counted for receptor-bound labelled TSH. Long-acting thyroid stimulator absorbing activity was expressed in terms of its ability to reduce the receptor-binding properties of Graves’ immunoglobulin G (IgG) using \(T = \frac{(T/C) \times 100}{C}\) where \(T = \frac{1}{25}\)I-labelled TSH specifically bound in the presence of LAA and normal pool serum minus \(125\)I-labelled TSH specifically bound in the presence of normal pool serum minus \(125\)I-labelled TSH specifically bound in the presence of Graves’ serum.

<table>
<thead>
<tr>
<th>TABLE 1. Typical levels of receptor-bound (^{125})I-labelled TSH obtained after incubation of Graves’ or normal pool serum with increasing amounts of soluble thyroid extract (0–500 (\mu)l). The increase in binding observed with increasing amounts of long-acting thyroid stimulator absorbing activity (LAA) is due to increased protein precipitation with polyethylene glycol during separation of bound and free labelled TSH. Values are means (\pm) S.D.; (n = 3). See Materials and Methods for experimental details</th>
</tr>
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<tbody>
<tr>
<td>Binding of (^{125})I-labelled TSH (%)</td>
</tr>
<tr>
<td>0 (\mu)l</td>
</tr>
<tr>
<td>Extract + Graves’ serum</td>
</tr>
<tr>
<td>Extract + normal pool serum</td>
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<tr>
<td>(T/C)</td>
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<td>((T/C) \times 100)</td>
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<td>((1 - (T/C)) \times 100)</td>
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\(T, 125\)I-labelled TSH specifically bound in the presence of LAA and normal pool serum minus \(125\)I-labelled TSH specifically bound in the presence of LAA and Graves’ serum.

\(C, 125\)I-labelled TSH specifically bound in the presence of normal pool serum alone minus \(125\)I-labelled TSH specifically bound in the presence of Graves’ serum alone.

serum alone minus $^{125}$I-labelled TSH specifically bound in the presence of Graves' serum alone.

In some instances, LAA activity was expressed as a percentage neutralization of Graves' serum, using $(1-\frac{T}{C}) \times 100$. Typical results are shown in Table 1.

**Bioassay of LAA**

This was carried out as described previously (Smith, 1971) using the mouse bioassay (McKenzie, 1958).

**Affinity chromatography**

Ligands were coupled to CNBr-activated Sepharose 4B (Pharmacia (Great Britain) Ltd, Hounslow, Middx) according to the manufacturer's recommended procedure, except for Sepharose–Concanavalin A (Con A) which was purchased ready coupled (Pharmacia). Bovine serum albumin and normal IgG were coupled at a protein concentration of 5–7 mg/ml gel. Human chorionic gonadotrophin (hCG; Pregnyl; Organon Laboratories Ltd, Morden, Surrey) was coupled at a hormone concentration of 1.5 mg/ml gel and TSH at a concentration of 50–60 units/ml gel. No leakage of TSH from the coupled TSH gel was detected by the TSH-receptor assay (Rickards et al. 1981). The columns (2 ml) were washed with 1% Lubrol 12A9 (ICI plc (Organics Division), Manchester) in Tris/NaCl and 3–5 ml samples of crude or partially purified LAA were applied to the columns at a flow rate of 2 ml/h at room temperature and eluted with Tris/NaCl. Fractions of 1.4 ml were collected and assayed for LAA content.

**Partial purification of LAA**

After centrifugation (100,000 g; 1 h; 4°C) LAA preparations (30 ml) were run on Sephacryl S-300 (Pharmacia) (5.5 x 39 cm) at 4°C in Tris/NaCl and the column eluant monitored for absorbance at 280 nm and LAA activity using the receptor assay. In some experiments fractions containing LAA were concentrated by ultrafiltration using a YM-10 membrane (Amicon Ltd, Stonehouse, Gloucs) and stored at -70°C.

**RESULTS**

A comparison of LAA measurements using the receptor assay and bioassay is shown in Fig. 1 and it can be seen that they were in good agreement. All subsequent studies were therefore carried out using the receptor assay. Repeat analyses indicated that the system was reproducible and that the water-soluble activity was heat-labile (Fig. 2). Absorption of receptor antibody activity was similar when water-soluble or particulate thyroid preparations were used and water-soluble extracts of human placenta prepared in an identical way to LAA showed no LAA activity. Sera from different patients having similar levels of TSH-receptor antibody activity appeared to show different degrees of reactivity with LAA (Fig. 3).

When LAA was fractionated on Sephacryl S-300 most of the activity was associated with relatively low molecular weight proteins (Fig. 4a), although some LAA (~30%) was eluted in the void volume. Further purification of the lower molecular weight LAA was not achieved on re-running on the S-300 column (Fig. 4b).

When crude and partially purified LAA were
figure 2. Comparison using receptor assay of long-acting thyroid stimulator absorbing activity (LAA) made on separate occasions. Samples of serum (50 µl) from a single patient with Graves' disease were incubated with increasing amounts of different crude LAA preparations on different occasions. Each symbol represents a different LAA preparation. The effects of heat-inactivated (30 min; 56 °C) LAA are also shown (broken line).

figure 3. Effect of long-acting thyroid stimulator absorbing activity on different sera from patients with Graves' disease. Samples of serum (50 µl) from patients with Graves' disease (with similar levels of TSH-receptor antibody) were incubated with increasing amounts of a water-soluble thyroid extract and then assayed for receptor antibody content. Each symbol represents serum from a different patient. All analyses were carried out using the receptor assay.

figure 4. Gel filtration of long-acting thyroid stimulator absorbing activity (LAA). (a) LAA (40 ml) run (40 ml/h at 4 °C) on a Sephacryl S-300 column (5.5 × 39 cm) in Tris/NaCl buffer. (b) The fractions containing the lower molecular weight LAA were pooled as shown by the solid bar in (a) and re-run on the same column. LAA activity is shown by the broken line and absorbance at 280 nm by the solid line. The elution profiles are typical of five separate experiments. All analyses were carried out using the receptor assay.

applied to columns containing Sepharose linked to BSA, normal IgG or hCG, the LAA was recovered unretarded in the elute fractions (Fig. 5a and b). The LAA content of these fractions was destroyed on heating (56 °C; 30 min). However, both crude and partially purified LAA preparations were absorbed by Sepharose-TSH and Sepharose-Con A (Fig. 5c and d). In preliminary experiments with Sepharose-TSH, material with TSH-like activity eluted from the column with the applied sample. This was surprising in view of the fact that the column had been thoroughly checked for TSH leakage before use. This TSH-like activity interfered with the subsequent assay for LAA. However, its release was prevented by addition of phenyl methyl sulphonyl fluoride (final concentration
DISCUSSION

The results described in this paper indicate that the TSH-receptor antibody assay could be used to detect LAA. The method appeared to give quantitatively similar results to those obtained with the mouse bioassay but was far less technically demanding and time-consuming and did not require the use of large numbers of experimental animals.

Using the receptor assay it was possible to show that LAA was heat-labile and principally associated with the lower molecular weight (4S) fraction of thyroid proteins, in good agreement with previous studies using the mouse bioassay (Smith, 1970, 1971). The gel-filtration analysis of thyroid extracts shown in Fig. 4 indicated that some LAA activity was associated with proteins eluting in the void volume of the column and this could represent aggregates of 4S LAA or small membrane fragments which had been incompletely sedimented during centrifugation. Significant levels of LAA activity, however, were not associated with 19S thyroglobulin (the major protein peak) in apparent contrast to earlier studies (Berumen, Lobsenz & Utiger, 1967; Smith, 1970, 1971) but the gel-filtration medium used in their studies (Sephadex G-200) was unable to separate effectively 19S thyroglobulin from higher molecular weight material. Thyroglobulin has been reported to influence assay responses in the mouse bioassay (Berumen et al. 1967; Dirmikis & Munro, 1973) but this type of effect does not seem to occur in studies with the receptor assay.

Affinity chromatography showed that LAA bound specifically to Sepharose–TSH and this provided good evidence that LAA contained a binding site for TSH as well as for TSH-receptor antibodies. The affinity of the LAA–TSH interaction was, however, lower than that between detergent-solubilized TSH receptors and TSH. This was evident from preliminary studies using columns of Sepharose–TSH containing a much lower concentration of hormone (4 units/ml gel), which readily absorbed the detergent-solubilized receptors but did not absorb LAA. The relatively low affinity of the LAA–TSH interaction could explain, in part at least, previous difficulties in demonstrating an interaction between them (Dawes, Petersen, Rees Smith & Hall, 1978).

Studies with columns of Con A–Sepharose indicated that they absorbed LAA in a similar fashion to detergent-solubilized TSH receptors (Rees Smith, McLachlan, Ginsberg et al. 1981), showing that both preparations contained sugar groups. Evidence for the glycoprotein nature of the TSH receptor has also been reported recently by Czarnocka, Gardas & Nauman (1981).

Our results indicate therefore that although LAA and the TSH receptor are hydrophilic and amphiphilic proteins respectively, both contain sugar groups and binding sites for TSH and for TSH-receptor antibodies. This suggests that LAA is a fragment of the receptor resulting from proteolytic cleavage at a point

![Diagram](https://example.com/diagram.png)

**FIGURE 5.** Affinity chromatography of long-acting thyroid stimulator absorbing activity (LAA) on columns of Sepharose–human chorionic gonadotrophin (hCG) and Sepharose–TSH. Crude (a and c) and partially purified (b and d) preparations of LAA were applied to columns of Sepharose–hCG (a and b) and Sepharose–TSH (c and d). Samples (300 µl) of the eluting fractions (1-4 ml) containing material not retarded on the columns were assayed for LAA (using the receptor assay) before (●) and after (○) heating at 56 °C for 30 min. The neutralization of Graves' serum attainable with 300 µl of the unfractionated preparations is indicated by the horizontal broken lines. The results shown are typical of two separate experiments in the case of crude LAA and three separate experiments in the case of partially purified LAA.

0.1 mmol/l) to the applied LAA preparations and this procedure was used in all subsequent experiments.
close to the outer surface of the thyroid cell plasma membrane as previously proposed by Dawes et al. (1978). However, the mechanisms involved in this cleavage process or its role in the action of TSH and TSH-receptor antibodies are not clear at present.

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REFERENCES


