A sensitive method for assaying thyroid stimulating immunoglobulins of Graves’ disease: use of the guanyl nucleotide-amplified thyroid adenylate cyclase assay

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Abstract. The purpose of this study was to develop and validate a sensitive method for evaluating adenylate cyclase stimulation by thyroid-stimulating antibodies (TSAb), based on the measurement of thyroid membrane adenylate cyclase activity in the presence of a non-hydrolyzable GTP analogue, guanyl-5’-yl imidodiphosphate (Gpp(NH)p).

The addition of Gpp(NH)p (10⁻⁵ M) produced a 10-fold increase of the sensitivity of the system for both TSH and TSAb. Immunoglobulin preparations from sera of 30 patients with Graves’ disease were tested for the adenylate cyclase stimulation either in the presence or in the absence of Gpp(NH)p: a significant stimulation was observed in 27/30 patients when the GTP analogue was added to the system, while only 20/30 patients were positive in the absence of the nucleotide. The advantage of Gpp(NH)p addition was also evident in a large series which included 57 patients with Graves’ disease, 15 with Hashimoto’s thyroiditis or primary myxoedema and 22 normal subjects. In fact, 88% of patients with Graves’ disease resulted positive, while no significant stimulation was elicited by Hashimoto’s thyroiditis, primary myxoedema and by normal immunoglobulins.

The sensitivity achieved in our system which employs thyroid plasma membranes was similar to that obtained by other investigators with the use of thyroid slices or thyroid cells in primary culture. Furthermore, methods based on thyroid plasma membranes are supposed to have a better reproducibility, since the same tissue preparation, if appropriately stored, may be used in several different tests.

Thyroid stimulating antibodies (TSAb) present in sera of patients with Graves’ disease have been shown to stimulate thyroid activity through an activation of adenylate cyclase, producing an increase of cAMP (Kaneko et al. 1970; Yamashita & Field 1972; Orgiazi et al. 1976; Bech & Madsen 1978; Macchia et al. 1981). Thyroid stimulation by TSAb may be evaluated by methods using human thyroid slices, cells or plasma membranes (Zakarija et al. 1980; Toccafondi et al. 1980; Orgiazi et al. 1976; Bech & Madsen 1978; Macchia et al. 1981).

The latter manner has the advantage that it can be used for a longer period of time if appropriately stored in liquid nitrogen or lyophilized. However, at present, methods employing thyroid plasma membranes (Orgiazi et al. 1976; Bech & Madsen 1979; Macchia et al. 1981; Karlsson et al. 1981) appear to have a lower sensitivity compared with both thyroid slices (Zakarija et al. 1980) and cells (Toccafondi et al. 1980). The addition of guanyl-5’-yl imidodiphosphate (Gpp(NH)p), a non-hydrolyzable analogue of GTP, has been advantageously...
used to increase the sensitivity of the plasma membrane adenylate cyclase system in glucagon-liver membranes (Iyengar et al. 1980) and PTH-renal cortical membranes (Nissenson et al. 1981). This prompted us to develop an assay with high sensitivity for TSAb by adding Gpp(NH)p to thyroid plasma membrane preparations.

The aims of this study were: i) to define the optimal conditions for the use of Gpp(NH)p in the human thyroid plasma membrane system, ii) to assess the extent of the increase of sensitivity in TSH and TSAb assay, using Gpp(NH)p, for both TSH and Graves’ IgG, iii) to demonstrate the usefulness of the Gpp(NH)p amplified assay in the measurement of TSAb.

Materials and Methods

Materials
Adenosine-5'-triphosphate, creatinephosphate and creatinekinase were purchased from Boehringer Mannheim, Mannheim, West Germany. Guanyl-5'-yl imidodiphosphate (Gpp(NH)p) was purchased from International Chemical and Nuclear Corp. (I.C.N.), USA. Bovine TSH was supplied by Armour Pharmaceutical Co., Phoenix, Arizona, USA. The SQ 20009 phosphodiesterase inhibitor (Carayon et al. 1978) was a gift from Squibb Laboratories, New Brunswick, NJ, USA.

Immunoglobulin preparation
Immunoglobulin G (IgG) was prepared, as previously described (Fenzi et al. 1978), by a modification of the DEAE Sephadex procedure (Baumstark et al. 1964), from patients with untreated Graves’ disease (G1gG), Hashimoto’s thyroiditis, primary myxoedema or from normal subjects (N1gG). The thyroid status was assessed by standard clinical and laboratory criteria. The diagnosis of Graves’ disease was based on the presence of: thyrotoxicosis, diffuse goitre with vascular bruit, mild or more severe signs of infiltrative ophthalmopathy, elevated thyroid radiiodine uptake and circulating anti-thyroid antibodies. Protein concentration was determined by the method of Lowry et al. (1951).

Plasma membrane preparation
Normal human thyroid tissue was obtained during operation from the extranodular tissue of a large multinodular goitre. Plasma membranes were then purified from the pellet obtained from thyroid homogenate between 300 × g and 30 000 × g by a discontinuous sucrose gradient centrifugation (Carayon et al. 1978, 1979, 1980). The same thyroid plasma membrane preparations were also tested after lyophilization. All the assays for adenylate cyclase activity were performed using the same plasma membrane preparation, obtained from a single thyroid gland processed no more than 30 min after surgery.

Assay of adenylate cyclase activity
If not otherwise indicated, bovine TSH or IgG, dissolved in 20 mM Tris-HCl buffer pH 7.8 bovine serum albumin 0.1%, were added to the incubation medium containing an ATP-regenerating system and the SQ 20009 phosphodiesterase inhibitor, as previously reported (Carayon et al. 1980). The optimum of adenylate cyclase activity was obtained with a Mg²⁺/ATP ratio of 4. The reaction was started by the addition of the membrane preparation, carried out at 34°C, and stopped by 1.9 ml of a cold methanol-ethanol mixture: using this mixture a nearly

![Fig. 1](https://example.com/fig1.png)

Effect of increasing amounts of Gpp(NH)p on adenylate cyclase activity of human thyroid plasma membranes. Each point is the mean ± SD of 4 determinations. This experiment was performed with the same human thyroid plasma membrane preparation. The incubation time was 60 min.
Fig. 2.
Basal and TSH (300 µU/ml) or IgG (5 mg/ml) stimulated adenylate cyclase activity in the absence or presence of 10⁻⁵ M Gpp(NH)p, as a function of time. Each point represents the mean of duplicates. GIgG = IgG from Graves' patients. NlG = IgG from normal subjects. This experiment was performed with the same human thyroid plasma membrane preparation.

Results

Assay conditions
Gpp(NH)p up to the concentration of 10⁻⁵ M was shown to increase the activity of adenylate cyclase in human thyroid plasma membranes (Fig. 1).

Larger amounts of Gpp(NH)p did not further increase the enzyme activity, being rather less effective. On the basis of these results subsequent experiments were performed with a concentration of the GTP analogue of 10⁻⁵ M.

Studies on the molarity and pH of the buffer (data not shown) demonstrated that the optimal molarity was 2 x 10⁻² M; the buffer pH of 7.8 was selected since higher or lower pH values markedly decreased both basal and TSH-stimulated adenylate cyclase activity in the presence of Gpp(NH)p.

Kinetic studies were performed in order to evaluate the optimal incubation time for stimulation of adenylate cyclase activity in the presence of Gpp(NH)p. The enzyme activity was measured at complete recovery of generated cAMP with precipitation of ATP was obtained. Adenylate cyclase activity was expressed as the production of cAMP/mg of membrane protein/min. cAMP was estimated by a sensitive and specific radioimmunoassay (Becton Dickinson cAMP kit). When patients' IgG was tested for adenylate cyclase activity, the control was represented by NlG at the same concentration.

Fig. 3.
Potentiation of TSH or IgG stimulation of adenylate cyclase in human thyroid plasma membranes by Gpp(NH)p. For convenience, the scale of ordinate after addition of Gpp(NH)p was reduced by a 3.2 factor. Gpp(NH)p was added at the concentration of 10⁻⁵ M. GlG = IgG from Graves' patients. This experiment was performed with the same human thyroid plasma membrane preparation. The incubation time was 120 min.
Effect of Gpp(NH)p on dose-response curves of TSH and GIGG

The effect of increasing amounts of bovine TSH and of a GIGG preparation with high thyroid stimulating potency in the absence or presence of Gpp(NH)p is illustrated in Fig. 3. In the absence of

Table 1.
Adenylate cyclase stimulating activity of immunoglobulins from patients with Graves’ disease (GIGG) in absence or presence of Gpp(NH)p.

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Results are expressed as per cent of adenylate cyclase activity in presence of IgG from normal controls. Values higher than 120% were significantly positive.

* Indicatives the values of adenylate cyclase stimulation which were negative in absence of Gpp(NH)p and became positive after the addition of the synthetic nucleotide.

the different time intervals either in basal conditions or after the addition of 300 µU/ml of bovine TSH, 3 mg/ml of NlG or 3 mg/ml of GIGG (Fig. 2). Parallel studies were carried out in the presence of 10-5 M Gpp(NH)p. The production of cAMP increased linearly during the first 30 min. Thereafter in the absence of Gpp(NH)p the plateau was reached after 60 min, whereas in its presence it was delayed for over 120 min. This finding prompted us to perform the guanyl nucleotide assay using an incubation time longer (120 min) than that used previously (Macchia et al. 1981) in the absence of Gpp(NH)p.

Similar results were obtained when lyophilized human thyroid plasma membranes were used.

Fig. 4.
Comparison of adenylate cyclase activation produced by TSH (○, Δ) or Graves’ IgG (GIGG) (o, Δ) in the absence (circles) or presence (triangles) of Gpp(NH)p 10^-5 M. Results are expressed as a percentage of maximal adenylate cyclase stimulation. This experiment was performed with the same human thyroid plasma membrane preparation. The incubation time was 120 min.
Adenylate cyclase activity measured by the guanyl nucleotide-amplified assay in different groups of patients. Results are expressed as a percentage of adenylate cyclase activity in the presence of 3 mg/ml of a pool of normal control IgG preparations. The horizontal dotted line indicates 2 SD above the mean of normal subjects. This experiment was performed with the same human thyroid plasma membrane preparation. The incubation time was 120 min.

Gpp(NH)p the dose-response curves showed a significant increase in cAMP production both with TSH and GIgG at concentrations of 30 μU/ml or 0.1 mg/ml, respectively. In the presence of Gpp(NH)p the amounts of stimulators needed for a significant effect were decreased to 3 μU/ml for TSH and 0.03 mg/ml for GIgG. As illustrated in Fig. 4, the nucleotide produced a leftward shift in the dose-response curves for both TSH and GIgG, thus indicating a higher sensitivity.

Measurement of the stimulating activity of IgG from patients with thyroid autoimmune disorders, using the nucleotide-amplified adenylate cyclase assay

Table 1 shows the effect on adenylate cyclase activity elicited by IgG preparations from 30 patients with Graves' disease in the absence or in the presence of Gpp(NH)p. Results are expressed as a percentage of the cyclase activity in the presence of equal amounts of normal IgG. The comparison of the results obtained showed that GIgG significantly stimulated thyroid adenylate cyclase in 20/30 cases (66.7%), in the absence of Gpp(NH)p; when the nucleotide was added, the number of GIgG able to stimulate the enzyme activity increased to 27/30 cases (90%), confirming a higher sensitivity of the present method.

The measurement of adenylate cyclase activity in the presence of Gpp(NH)p was performed in a large series of 57 unselected patients with Graves' disease, 15 with Hashimoto's thyroiditis or primary myxoedema and 22 normal subjects (Fig. 5). None of the normal subjects or the patients with Hashimoto's thyroiditis or primary myxoedema showed any significant stimulation of the enzyme activity. In contrast 87.8% of Graves' patients resulted positive, indicating a great specificity of this nucleotide-amplified assay.

Discussion

In this study we have developed a method for measuring the adenylate cyclase stimulation in human thyroid plasma membranes, based on the addition of Gpp(NH)p, which can be usefully employed for the assessment of TSAb activity.

The addition of Gpp(NH)p has been shown to enhance both basal and stimulated thyroid adenylate cyclase activity. The guanyl nucleotide-amplified system exhibited a higher sensitivity for TSH, when compared to the assay performed in the absence of Gpp(NH)p, and allowed the detection of as little as 3 μU/ml of bovine TSH. An increased sensitivity was also demonstrated for TSAb. Comparison of the results obtained with the same thyroid plasma membrane preparation in the presence or in the absence of Gpp(NH)p showed that in a series of 30 patients with Graves' disease the percentage of positivity increased from 66.7% to 90% in the guanyl nucleotide-amplified system.
Due to the higher basal activity in the Gpp(NH)\textsubscript{p} assay, the stimulability (times the basal activity) elicited by the highest doses of TSH or by the most potent Graves' IgG was reduced.

GTP is known to potentiate the effect of thyroid adenylate cyclase stimulators (Wolff & Cook 1973), and it appears to be necessary for the full expression of adenylate cyclase activity. The progressive decrease of adenylate cyclase activity commonly observed in plasma membrane preparations of thyroid and other tissues may be due to the hydrolysis of endogenous GTP produced by membrane associated GTPases (Levitzky 1978; Rodbell 1980; Iyengar et al. 1980; Limbird 1981). This problem may be overcome by the addition of Gpp(NH)\textsubscript{p}, a non-hydrolyzable analogue of GTP, which, similarly to GTP, has been found to increase the response to thyroid adenylate cyclase stimulators (Wolff & Cook 1973). A similar effect of this compound has been demonstrated in other systems including glucagon-liver adenylate cyclase (Iyengar et al. 1980) and PTH-renal cortical adenylate cyclase (Nissenson et al. 1981).

The clinical usefulness of the present method was assessed by assaying TSAb activity in IgG preparations from 57 untreated patients with unselected Graves' disease, 15 with Hashimoto's thyroiditis or primary myxoedema and 22 normal subjects. Positive responses were found in 88% Graves' patients, while negative results were obtained in the other 2 groups. Negative results obtained in some patients with Graves' disease may be due to the small concentration of IgG used, in order to avoid a non-specific effect of normal IgG (Carayon et al. 1980). Another explanation is that the concentration of TSAb IgG in sera of some Graves' patients is so small that it cannot be measured even by the use of this sensitive assay.

Previous reports indicated that human thyroid plasma membranes were less sensitive than human thyroid slices or cells in the measurement of TSAb activity. Orgiazi et al. (1976) reported that using 'crude' thyroid plasma membranes only 60% of IgG preparations from Graves' patients was able to stimulate adenylate cyclase activity. Seventy-one to 82% of positivity has been reported by other investigators in more recent studies using thyroid plasma membranes (Bech & Madsen 1979; Macchia et al. 1981; Karlsson et al. 1981). A sensitivity similar to that achieved in the present study has been obtained only when TSAb adenylate cyclase stimulatory activity was measured in human thyroid slices or human thyroid cells in primary culture (Zakarija et al. 1980; Toccafondi et al. 1980). Most recently a continuously cultured line of normal rat thyroid cells has also been shown a sensitive system for TSAb assay (Vitti et al. 1982).

The method described in this paper appears to have the same sensitivity as compared to slices or cells. Thyroid membranes have the great advantage that they can be stored in liquid nitrogen or lyophilized for long periods without losing their biological activity. Therefore, different assays can be performed with the same membrane material, thus reducing the inter-assay variations. Moreover, there is no need for a fresh thyroid preparation for each assay.

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References


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