A FACTOR IN CYTOSOL ENHANCES STIMULATION OF MEMBRANE ADENYLATE CYCLASE FROM HUMAN THYROID BY THYROTROPHIN AND THYROID STIMULATING IMMUNOGLOBULINS

C. A. OLLIS, S. TOMLINSON, S. MAC NEIL, A. CRAWFORD and D. S. MUNRO*

Department of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School, Beech Hill Road, Sheffield, S10 2RX and **Department of Medicine, University of Sheffield Medical School, The Northern General Hospital, Sheffield, S5 7AU, England*

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1. Introduction

Thyroid adenylate cyclase activity has been shown to increase in response to thyrotrophin (TSH), leading to a rapid increase in intracellular adenosine 3′,5′-cyclic monophosphate (cyclic AMP), which is thought to mediate the intracellular response to TSH. This has been demonstrated in both thyroid slices [1,2] and in particulate membrane preparations [3,4]. Two types of thyroid stimulating autoantibodies (TSIg) have been detected in the serum of patients with Graves' disease, long-acting thyroid stimulator (LATS) [5] and LATS-protector (LATS-P) [6]. These immunoglobulins may also stimulate adenylate cyclase resulting in cyclic AMP accumulation in thyroid cells. The stimulation of cyclic AMP production by TSIg has been clearly shown in thyroid slices [1,2,7,8] but there are conflicting reports of the effects of TSIg on the stimulation of adenylate cyclase in human thyroid membrane preparations [2,9,10].

We have shown that there is a factor in cell cytosol which enhances hormone-stimulated adenylate cyclase [11]. This factor shows little evidence of tissue specificity as cytosols derived from different tissues stimulated adenylate cyclase activity as much as cytosols derived from the tissue of origin. The mode of action of this factor has been shown to be similar to the action of guanosine triphosphate (GTP), although characterisation and partial purification of the factor have established that the factor is not free GTP [12].

Here we have investigated the effect of human thyroid cytosol, human platelet cytosol and GTP on the ability of TSH and TSIg to stimulate human thyroid membrane adenylate cyclase activity.

2. Methods and materials

Human thyroid tissue was obtained at surgery and kept at 0–4°C. The tissue was chopped with a razor and then homogenised in 10-times its volume of 0.25 M sucrose 20 mM Tris/HCl (pH 7.4) using a Polytron homogeniser for 3 × 10 s at medium speed. Further homogenisation was carried out with 5 strokes of a loose fitting Dounce homogeniser. After centrifugation of the homogenate at 800 × *g* to remove tissue debris, the supernatant was further centrifuged at 10 000 × *g* for 15 min. The resulting pellet was either resuspended in 20 mM Tris/HCl (pH 7.4) with 0.25 M sucrose for immediate assay or stored at −70°C in 0.25 M sucrose, 0.2 mM $MgCl_2$, 1 mM EGTA in 25 mM Tris/HCl (pH 7.4) with 10% (v/v) dimethylsulphoxide.

Adenylate cyclase activity was measured as in [13], and [^{32}P]cyclic AMP was isolated according to [14]. Membrane protein concentrations were determined by method in [15]. Results are expressed as the mean ± SEM of 3 experiments.

Isolated thyroid cells were prepared from non-toxic goitre obtained at surgery. The tissue was chopped finely and washed with calcium magnesium free Dulbecco's medium and then sequentially digested for four 30 min periods over 2 h using 0.25% dispase. Isolated cells were collected by centrifugation at 500 × *g* for 10 min, washed twice with Ca, Mg-free Dulbecco's medium and the final cell pellet resuspended

in 199 medium containing 15% foetal calf serum, 0.02 M *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulphonic acid (Hepes) (pH 7.4) and 0.02% $NaHCO_3$, plated in 90 cm^2 Petri dishes and grown to confluency in the same medium.

Thyroid cytosol was prepared from cells which had been maintained in culture for 1 week, and passaged once. Petri dishes were washed free of culture medium with sucrose buffer containing 0.25 M sucrose, 1 mM EDTA and 15 mM Tris/HCl (pH 7.4). The cells were scraped from the plate surfaces, suspended in the same buffer (0.5 ml/90 cm^2 plate) and frozen with a mixture of solid CO_2 and ethanol and then thawed at 37°C. This freeze–thawing was repeated twice to lyse the cells. Centrifugation of the resulting whole cell lysate at 10 000 × *g* for 10 min yielded a 'cytosol' preparation which was stable at –20°C.

Platelet cytosol was prepared from 30 ml fresh blood mixed with 3 ml 3.8% citrate (w/v) 0.1 M EDTA and spun for 10 min at 150 × *g* at room temperature yielding platelet rich plasma (PRP) which was removed with a plastic pipette. PRP was further spun at 500 × *g* for 10 min at room temperature and the platelet pellet resuspended in 15 mM Tris/HCl (pH 7.4) with 1 mM EDTA and 140 mM NaCl. After a second spin at 500 × *g* for 10 min at room temperature the pellet was resuspended in ~2–3 ml of 0.25 M sucrose 10 mM Tris/HCl (pH 7.4) at 40°C and lysed by freeze–thawing as described. Lysates were centrifuged at 50 000 × *g* for 30 min to yield a cytosol preparation which was stored at –20°C.

LATS and LATS-P were obtained from sera from patients with Graves' disease. Immunoglobulin G preparations of LATS were prepared from serum samples obtained from thyrotoxic patients [16]. The potency of LATS-IgG was estimated by the bioassay [17] as modified [18] and was found to be 760 mU/μl Medical Research Council Reference standard B for LATS.

[α-^{32}P]ATP (0.5–30 Ci/mmol) and cyclic[α-^{3}H]-AMP (20–30 Ci mmol) were obtained from the Radiochemical Centre, Amersham. Bovine serum albumin (fraction V, powder) was obtained from Armour Pharmaceutical Co., Eastbourne and all other biochemicals from Boehringer Mannheim, London. Medium 199 was obtained from Gibco.

Thyroid stimulating hormone (bovine pituitary) was obtained from Sigma, London. For experimental use, freshly weighed amounts of TSH were dissolved in a small known volume of assay buffer and used immediately.

3. Results

Platelet cytosol was used in the initial experiments because of the difficulties of obtaining a thyroid cytosol preparation which was not contaminated with extracellular fluid. Factors present in extracellular fluids have a non-specific inhibitory effect on adenylate cyclase activity [11]. In subsequent experiments thyroid cells were maintained in culture and thyroid cytosol prepared from these cells. Neither platelet nor thyroid cytosol had any intrinsic adenylate cyclase activity.

Preliminary experiments showed that the ability of platelet cytosol to enhance TSH-stimulated adenylate cyclase activity was greater if the membrane pellet was resuspended directly in cytosol and both were present at the initiation of the response rather than if the membranes and cytosol were added to the assay separately. Consequently, in the following experiments, the membrane pellet was resuspended directly in buffer, cytosol or GTP and used to start the reaction. In the limited number studied, membranes prepared from non-toxic goitres were more sensitive to TSH than membranes from toxic goitres and normal thyroid tissue. The following results are all based on preparations derived from non-toxic goitres. Figure 1 shows the effect of GTP (10^{-6} M) and platelet cytosol on the response of the membrane preparation to TSH. Membranes in buffer showed an ~6-fold stimulation of adenylate cyclase activity over basal in response to the maximum TSH concentration used. In the presence of GTP and in the presence of cytosol there was again a 6-fold stimulation of adenylate cyclase activity in response to TSH, although the rate of cyclic AMP production was much greater in both cases. Neither GTP nor cytosol significantly altered the fluoride-stimulated enzyme activity. Preliminary experiments indicate that the cytosol factor has no effect on the concentration of TSH needed to cause half maximal adenylate cyclase response.

Table 1 shows that platelet cytosol increased the response to TSH and only in the presence of cytosol was there a significant response to LATS-IgG. The

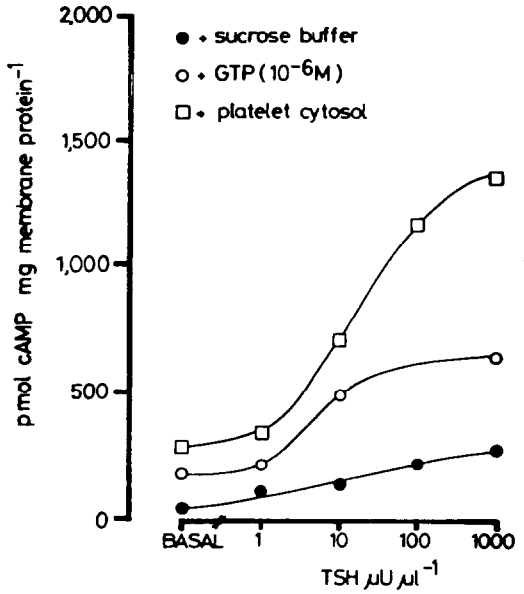

Fig.1. Adenylate cyclase activity in membranes obtained from a non-toxic goitre in response to TSH (1–1000 μU/μl). The period of incubation with TSH was 10 min at 37°C. Effect of platelet cytosol (20 μg cytosol protein/100 μl incubation mixture) and GTP (10^{-6} M) on the rate of production of cyclic AMP.

effect of the platelet cytosol could not be mimicked by GTP. These results have since been repeated in a non-toxic goitre obtained from another patient. Table 2 shows the effects of thyroid cytosol and of thyroid cytosol that had been extensively dialysed on the response to TSH and LATS-IgG. Cytosol increased the response to TSH and only in the presence of cytosol was there a significant increase in adenylate cyclase activity in response to LATS-IgG. This effect of cytosol was retained on dialysis.

4. Discussion

The action of the thyroid stimulating immunoglobulins on the thyroid has been well studied with regard to release of ^{125}I from a pre-labelled human thyroid in vivo [19], uptake of ^{131}I by the thyroid in thyrotoxicosis [20] and colloid droplet formation in human thyroid slices [21] in response to TSIg. The action of TSIg has been studied in human thyroid membranes [22] using binding studies in which TSIg displaced TSH from membranes; such studies imply that the binding sites of TSH and TSIg may be identical or closely adjacent. The binding of TSIg and subsequent stimulation of adenylate cyclase by TSIg in human thyroid slices has been shown [1,2,7,8] but the situation in thyroid membranes is not so clear. Binding of TSIg has been clearly demonstrated in thyroid membranes [22] but the activation of adenylate cyclase has not been consistent suggesting that the binding of TSIg is not always synonymous with the stimulation of adenylate cyclase.

The discrepancy in the results of studies of membranes and slices can possibly be explained by the loss of a factor(s) during the preparation of thyroid membranes, which is present in slices. We have shown that a factor in cell cytosol which is not hormone, species or tissue specific enhances hormone-stimulated adenylate cyclase activity [12]. Consequently, the

Table 1
The effect of GTP (10^{-6} M) and platelet cytosol (20 μg cytosolic protein/100 μl incubation mixture) on the adenylate cyclase response to TSH and LATS-IgG in a membrane preparation from a non-toxic goitre

	(pmol cAMP produced/mg membrane protein)		
	+ Sucrose buffer	+ Platelet cytosol	+GTP
Basal	138 ± 4.2	265 ± 17	134 ± 11
TSH (1 mU/μl)	957 ± 25	2147 ± 17	911 ± 46
LATS-IgG	173 ± 33	626 ± 21[a]	160 ± 31

[a] Significantly different from basal value ($P < 0.001$) by t test

Incubation period of 10 min at 37°C with both TSH (1 mU/μl) and LATS-IgG (760 mU/μl MRC reference standard B for LATS). The mean ± SEM (n=3) are shown

Table 2
The effect of thyroid cytosol and dialysed thyroid cytosol on the adenylate cyclase response to TSH (1 mU/μl) and LATS-IgG (760 mU/μl)

	(pmol cAMP produced/mg membrane protein)		
	+ Sucrose buffer	+ Thyroid cytosol	+ Dialysed thyroid cytosol
Basal	38.8 ± 3.0	73.7 ± 4.7	51.9 ± 3.0
TSH (1 mU/μl)	367 ± 29.0	640 ± 4.3	572 ± 11.7
LATS-IgG	33.5 ± 5.0	111.2 + 2.3[a]	134 ± 3.3[b]

Significantly different from basal: [a] ($P < 0.01$); [b] ($P < 0.001$) by *t* test

Cytosol was dialysed for 24 h against several changes of sucrose buffer in which the cytosol was prepared. Incubation period of 10 min at 37°C. The mean ± SEM are shown

effects of human thyroid and platelet cytosols on the adenylate cyclase response to TSH and TSIg were studied. Our results indicate that a lack of response to TSIg may be due to the loss of some factor present in cell cytosol which occurs during the preparation of membranes. This factor is unlikely to be free GTP as the effect persisted after extensive dialysis of the cytosol and could not be mimicked by the addition of GTP to the assay.

In conclusion, the addition of a cell cytosol preparation to the membranes can affect human thyroid adenylate cyclase responsiveness to both TSIg and TSH in vitro and may be relevant to the action of TSIg in vivo.

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