THYROTROPHIN RECEPTOR BINDING, INTRACELLULAR CYCLIC AMP LEVELS AND IODINE RELEASE IN ISOLATED THYROID CELLS

Pamela M. POVEY, Bernard REES SMITH, Terry F. DAVIES and Reginald HALL
Departments of Medicine and Clinical Biochemistry, University of Newcastle upon Tyne, NE1 7RU, England

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1. Introduction

There is now considerable evidence that hormone–receptor interactions activate adenylate cyclase and the resulting increases in cyclic AMP levels mediate the effects of the hormone [1]. However, a study of corticotrophin (ACTH) stimulation of cyclic AMP production and steroidogenesis in isolated adrenal cells has suggested that cyclic AMP is not involved in the stimulation of steroidogenesis by small amounts of ACTH [2]. Similarly, human chorionic gonadotrophin (hCG)-stimulated testosterone production in dispersed interstitial cells appears to be independent of changes in cyclic AMP levels [3]. Scranton and Tong [4] have reported that thyrotrophin (TSH) causes a rapid stimulation of iodine release from isolated thyroid cells in the absence of stimulation of iodine uptake and in this paper. We describe a study of the relationship between TSH stimulation of iodine release, intracellular cyclic AMP levels and TSH receptor binding using isolated porcine thyroid cells. Since sera from patients with Graves' disease contain antibody molecules which mimic the effects of TSH by interacting with the TSH receptor [5], the effects of these Graves' immunoglobulins on the isolated thyroid cells have also been studied.

2. Methods

2.1. *Thyroid cell isolation and culture*

Porcine thyroid cells were isolated by trypsinisation of finely chopped tissue [6] and cultured on plastic supports in the presence of 0.4 mM dibutyryl cyclic AMP for 48 or 72 h [7]. During this period the cells formed 3-dimensional follicular structures which were capable of iodine organification [8]. Cells were removed from the plastic support after the culture period treatment with 3 mM EGTA [9]. The cells were washed and resuspended in minimal essential medium containing 10% foetal calf serum and 20 mM Hepes pH 7.4 (Medium H). Cell viability was determined using trypan blue dye exclusion [10] and found to be about 80%.

2.2. *Thyrotrophin receptor binding*

Highly purified bovine TSH (30 units/mg, generous gifts from Dr J. G. Pierce and Dr J. Fawcett) was labelled with ^{125}I to a specific activity of 160 μCi/μg and receptor purified using human thyroid membranes [11]. Studies with a highly sensitive cytochemical bioassay [12] indicated that the ^{125}I-labelled hormone showed identical biological activity to the unlabelled TSH. Harvested cells (2×10^6 in 0.25 ml of Medium H) were added to 0.25 ml of Medium H containing 15 000 cpm (60 pg) of labelled TSH, 10 nM KI and varying doses of unlabelled bovine TSH (1 unit/mg, a generous gift from the Armour Pharmaceutical Company, UK). After incubation at 37°C in a shaking water bath (150 cycles/minute) the reaction mixtures were diluted with 2.5 ml of ice cold Medium H and centrifuged for 1 min at $1500 \times g$. The supernatants were aspirated, the cell pellet washed once by resuspension in 2.5 ml of Medium H followed by re-centrifugation and the amount of ^{125}I in the pellet measured. All determinations were made in triplicate.

2.3. *Stimulation of ^{125}I release from the isolated thyroid cells*

Suspensions of harvested cells (4×10^6/ml) were incubated with $Na^{125}I$ (0.4 μCi/10^6 cells) for 20 min at 37°C with gentle shaking and then washed twice with Medium H to remove $Na^{125}I$ not taken up by the cells. About 6% of the ^{125}I added was taken up and this could be reduced to less than 0.3% by addition of $KClO_4$ to 10^{-3} M. The cells were resuspended in Medium H (8×10^6 cells/ml) and 250 μl aliquots immediately added to 250 μl of the same medium containing test material. During incubation at 37°C with shaking (150 cycles/min) the tubes were briefly removed from the water bath and the total amount of ^{125}I in each reaction mixture determined. The cells were then centrifuged at $1500 \times g$ for 1 min and the amount of ^{125}I in the cell pellet determined. All determinations were made in triplicate. Results were expressed as the % ^{125}I released in the presence of test material minus the % ^{125}I released in the presence of Medium H only or Medium H containing appropriate control material.

2.4. *Intracellular cycle AMP levels*

Isolated thyroid cells (8×10^6/ml) in 0.75 ml of Medium H were added to 0.75 ml of the same medium containing unlabelled bovine TSH and incubated with shaking at 37°C. After various times the mixtures (groups of 3 for each determination) were centrifuged ($1500 \times g$ for 1 min) and the cell pellets suspended at 0°C in 250 μl of 10 mM theophylline. The suspensions were heated at 85°C for 10 min. cooled to 0°C and centrifuged for 15 min at $1500 \times g$. Aliquots (2×50 μl) were determined for cyclic AMP by a competitive protein binding method [13]. Results were expressed as the amount of cyclic AMP produced in picomoles/2×10^6 cells.

2.5. *Preparation of immunoglobulins*

Immunoglobulins were precipitated from serum by addition of 3.75 M ammonium sulphate [11], dialysed exhaustively against 150 mM NaCl followed by 20 vol. of Medium H (without calf serum) and stored at −25°C.

3. Results

3.1. *Thyrotrophin receptor binding*

About 1% of the labelled TSH added to suspensions of thyroid cells was bound by the cells in the absence of unlabelled TSH. Significant inhibition of ^{125}I–TSH binding was observed with the addition of as little as 0.3 ng (10 μU) of unlabelled hormone. Labelled TSH binding was also inhibited by Graves' immunoglobulins (table 1). Large doses of unlabelled TSH (3000 ng) reduced labelled hormone binding to about 0.2% whereas similar amounts of ACTH or hCG reduced binding only slightly. The amount of label bound in the presence of 3000 ng of unlabelled hormone was taken as non-specific binding and all binding data were corrected for non-specific binding by this method. Labelled TSH binding to empty plastic tubes or plastic tubes containing mouse spleen lymphocytes was about 0.1% and this was not significantly reduced in the presence of large doses of unlabelled TSH. Figure 1 shows the relationship between the amount of TSH added to the cells and the amount of TSH bound (calculated by multiplying the total amount of hormone present by the fraction specifically bound).

Table 1
The effect of Graves' immunoglobulins on the binding of ^{125}I–TSH to isolated thyroid cells expressed as a (%) ± SE

Test material		^{125}I–TSH Bound (%)
Medium only		1.2 ± 0.1
Normal immunoglobulins (10 mg/ml)		1.0 ± 0.0
Graves' immunoglobulins (10 mg/ml)		
	Ch	0.7 ± 0.0[a]
	Wh	0.6 ± 0.1[b]
	Tu	0.7 ± 0.1[a]
	Ca	0.6 ± 0.1[b]

[a]Indicates results significantly different from normal immunoglobulins at $P = 0.05$ and [++] at $P = 0.01$.

Kinetics studies indicated that with a hormone concentration of 6.6 ng/ml and 4×10^6 thyroid cells/ml, TSH binding was half maximal at 2.5 min and maximal at 10 min (fig.2). Dissociation of bound labelled TSH was found to be half maximal at 4 min in the presence of excess (6 μg/ml) unlabelled TSH.

Scatchard analysis [14] of equilibrium binding data gave approximately linear plots. Two separate experiments gave association constants of 1.3 and 1.8×10^9 M^{-1}. The mean maximum TSH binding capacity of 2×10^6 cells was 100 pg which represented about 10^3 sites/cell.

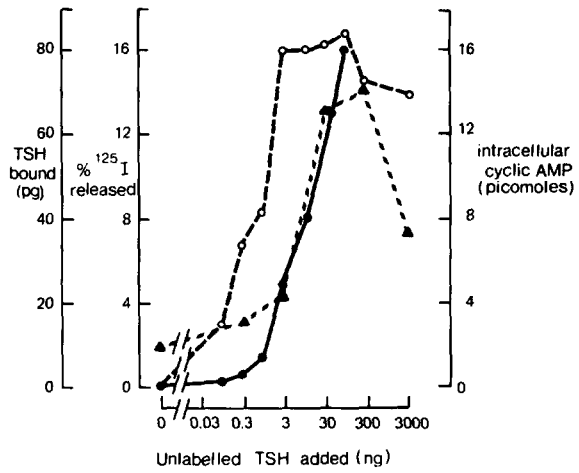

Fig.1. The relationship between TSH receptor binding (•), intracellular cyclic AMP levels (▲) and ^{125}I release (○) in isolated thyroid cells. Cell concentration 4 × 10^6/ml, reaction volume 0.5 ml, incubation time 10 min. Each point shown is a mean of closely agreeing triplicates. In the absence of unlabelled TSH (point 0 on TSH added axis) the amount of labelled hormone bound was 0.6 pg. In the presence of 0.1 ng of unlabelled TSH the amount of hormone bound (unlabelled + labelled) was 2 pg. The data were typical of several separate experiments.

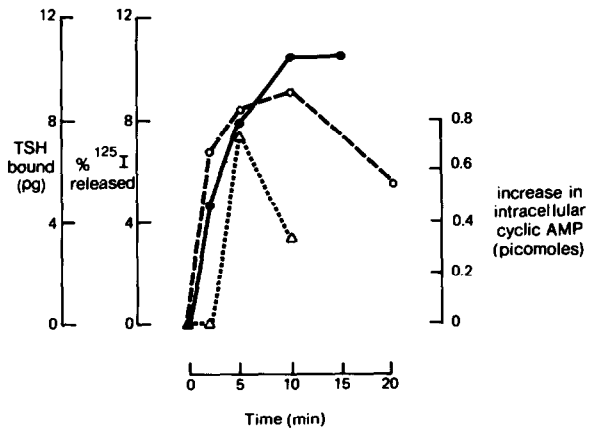

Fig.2. Kinetics of TSH receptor binding (•), changes in intracellular cyclic AMP levels (△), and ^{125}I release (○) in isolated thyroid cells. Cell concentration 4 × 10^6/ml, TSH concentration 6.6 ng/ml. Each point shows the mean of closely agreeing triplicates. The data were typical of several separate experiments.

3.2. *Intracellular cyclic AMP levels*

Detectable increases in intracellular cyclic AMP levels were observed with as little as 0.3 ng of TSH (fig.1). Half maximal increases were observed with 10 ng of hormone and maximal increases with 300 ng. Larger doses of TSH gave submaximal changes in cyclic AMP levels. Kinetic studies indicated that with 4 × 10^6 cells/ml and a TSH concentration of 6.6. ng/ml changes in cyclic AMP levels were undetectable at 2 min, maximal at 5 min and less than maximal at 10 min (fig.2).

3.3. *^{125}I release*

Detectable stimulation of ^{125}I release by the cells was observed with the addition of less than 0.3 ng of TSH (fig.1). Half maximal stimulation of ^{125}I release was observed with 1 ng of TSH and maximal stimulation with 3 ng. Analysis of the radioactive materials released from the cells by thin layer chromatography [15] indicated that about 90% of the ^{125}I released was in the form of $Na^{125}I$. Kinetic studies showed that with 4 × 10^6 cells/ml and a hormone concentration of 6.6 ng/ml half maximal stimulation of ^{125}I release occurred after less than 2 min and maximal stimulation after 10 min (fig.2). Dibutyryl cyclic AMP stimulated ^{125}I release from the isolated cells but somatostatin, ACTH and hCG were without significant effect (table 2).

Iodine release was also stimulated to a small, but significant, extent by immunoglobulins from a pool of normal serum (table 2). The immunoglobulins from only 2 (patients Su and He) out of the 6 Graves' sera studied stimulated ^{125}I release to an extent significantly greater than the normal pool. The effects of the active Graves' immunoglobulins showed similar kinetics to those of TSH.

4. Discussion

The interaction between TSH and TSH receptors on isolated thyroid cells was characterized by rapid association and dissociation reactions. Scatchard analysis of equilibrium binding data suggested the presence of a single population of non-interacting TSH binding sites with an association constant of about 10^9 M^{-1}. These results were in good agreement with a recent report by Verrier et al. [9].

Table 2
Stimulation of iodine release from isolated thyroid cells

Sample	Concentration	^{125}I Released (%)
TSH	0.2 nM	20.1 ± 0.9[b]
	20 nM	21.4 ± 0.9[b]
Dibutyryl cyclic AMP	0.2 mM	1.9 ± 0.9
	2 mM	14.2 ± 0.9[b]
ACTH	20 mM	2.1 ± 1.0
hCG	20 nM	2.4 ± 1.1
Somatostatin	350 nM	2.3 ± 1.5
	700 nM	0.0 ± 1.6
Immunoglobulins	10 mg/ml	
Normal pool		3.3 ± 0.7[a]
Graves' Ch		−0.7 ± 1.1
Wh		1.4 ± 0.9
Tu		4.4 ± 0.7[b]
Ca		0.5 ± 2.2
Su		10.9 ± 1.9[b]
He		7.1 ± 1.4[a]

The ^{125}I released was expressed as the difference between means of samples containing medium only and samples containing test material ± standard error of the difference. [a]Indicates significant release at $P = 0.05$ and [b]at $P = 0.01$. Only the immunoglobulins from patients Su and He stimulated ^{125}I release to an extent greater than the normal pool ($P = 0.05$).

Several studies have indicated that the effects of TSH on the thyroid are mediated by cyclic AMP [1,16] and our observations that dibutyryl cyclic AMP was able to stimulate ^{125}I release from isolated thyroid cells (table 2) was consistent with these earlier studies. Minimal detectable changes in receptor binding, intracellular cyclic AMP levels and ^{125}I release were observed with similar doses (about 0.3 ng) of TSH (fig.1). Although subsequent increases in intracellular cyclic AMP levels corresponded approximately with increases in receptor binding only small changes in these two systems corresponded with maximal stimulation of ^{125}I release.

The iodine released by the cells was mainly in the form of Na^{125}I indicating that little iodine organification had occurred. This was probably due to a lack of follicular structure in the porcine thyroid cell suspensions as such a structure has been shown to be necessary for extensive iodine organification in vitro [7]. Similarly, suspensions of bovine thyroid cells have been shown to organify only small amounts of iodine, particularly in the absence of TSH [17].

Kinetic studies indicated that 2 min after addition of TSH, stimulation of ^{125}I release was greater than half maximal, receptor binding slightly less than half maximal and any changes in intracellular cyclic AMP levels undetectable. Consequently considerable TSH stimulation of ^{125}I release occurred before any detectable changes in intracellular cyclic AMP levels and this might have indicated that cyclic AMP was not involved in mediating the stimulation of iodine release. However, dibutyryl cyclic AMP was able to mimic the effects of TSH on ^{125}I release and therefore, the data suggested that the effect of TSH on ^{125}I release was mediated by cyclic AMP but that only small changes in cyclic AMP concentration were involved.

Increases in intracellular cyclic AMP levels were consistently reduced by incubations longer than 5–10 min (fig.2). This effect could possibly have involved stimulation of intracellular phosphodiesterase activity by TSH in a way analogous to the effects of insulin on fat cells [18]. Prolonged incubation or large doses of TSH also resulted in a reduction in ^{125}I release, but this was probably due to stimulation of the apparently slower process of ^{125}I uptake.

Immunoglobulins from patients with Graves' disease interacted with the TSH receptor on isolated thyroid cells in such a way as to inhibit labelled TSH binding (table 1). This observation was in good agreement with previous studies on the binding of Graves' immunoglobulins and TSH to thyroid membranes [5,11] and isolated thyroid cells [19]. However the interaction between the antibody molecules and the isolated porcine thyroid cells did not appear to be particularly effective in causing cell stimulation as immunoglobulins from only 2 out of the 6 sera studied were capable of inducing significant stimulation of ^{125}I release (table 2).

Consequently the interaction of Graves' immunoglobulins with the porcine TSH receptor does not always lead to cell stimulation. This phenomenon may be related to the partial phylogenetic specificity of the thyroid stimulating activity of Graves' immunoglobulins originally described by Adams and Kennedy [20]. However, recently we have observed in a few patients with ophthalmic Graves' disease the presence of antibodies which interact with the human TSH receptor but are unable to stimulate thyroid function [21] and this can be considered analogous to our observations with the porcine TSH receptor.

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