ISOLATION OF TSH-RESPONSIVE HUMAN THYROCYTES –
ANALYSIS OF VARIOUS THYROID TUMOURS

By

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ABSTRACT

A method for the isolation of hormone-responsive thyrocyte suspensions from human tumours is described. Cells from normal and adenomatous thyroid tissue responded to thyroid stimulating hormone (TSH) by a concentration-dependent increase in cAMP. Bovine TSH usually produced half-maximal elevation of cAMP levels at concentrations between 2 and 4 mU/ml. Human TSH caused half-maximal stimulation at 3–8 times lower concentrations indicating considerable species specificity of the human TSH receptor. Other hormones were without influence except prostaglandin E1 (PGE1) which evoked moderate elevations of the cAMP levels.

Kinetic analyses of various thyroid tumours revealed marked differences in TSH induced increases in the intracellular cAMP levels with respect to the time course as well as to the extent of the maximal response. Theophylline, which by itself had a rather small effect on basal cAMP levels, acted synergistically with TSH in most cases, leading to values up to 20 times above the control values. The kinetics also indicated the existence in stimulated cells of two cAMP pools exhibiting different susceptibility to phosphodiesterase.

Cells from adenomatous goitres with hyperplasia exhibited significantly lower basal cAMP levels than thyrocytes from the other goitres and from normal tissue. Other classes of thyroid tumours also showed characteristic differences with regard to basal cAMP and degree of stimulation by TSH and theophylline.

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Tumour cells often retain their capacity for the special metabolic functions of the tissue from which they originate. However, partial or complete deletions in regulatory abilities is one of the first signs of cancerous deviation (cf. Pirot et al. 1961; Schorr et al. 1972; Anderson et al. 1974). Such deletions have also been described in thyroid tumours of the rat (cf. Macchia et al. 1972; D'Armi-ento & Monaco 1974) as well as in humans (Schorr et al. 1972; Demeester-Mirkine et al. 1976).

The aim of our analyses is to localize regulatory deletions in human thyroid tumours. Since most of the physiological effects of the thyroid stimulating hormone (TSH) are mediated by the cAMP system (see Schell-Frederick & Dumont 1970; Dumont 1971; Field 1975 for a review), we have analyzed as a first step of the regulatory sequence: Hormone binding → hormone response, the ability of human thyroid tumours to respond to TSH by an increase in intracellular cAMP.

Few human thyroid tumours have been analyzed previously for their ability to increase cAMP in response to TSH. In two analyses (cf. Schorr et al. 1972; Sand et al. 1976), only adenylate cyclase activity was measured in homogenates, while the other determinations were carried out with tissue slices (Burke & Szabo 1972; DeRubertis et al. 1971; Dumont et al. 1975). In slices, slow penetration of peptide hormones to the target cells and their possible destruction by liberated proteases usually leads to an unfavourable response with respect to time and hormone concentrations (cf. Swillens et al. 1976). Furthermore, because of the heterogeneous structure of the tumourous tissue along with the drastic differences in the content of connective tissue, blood constituents, and the size of the follicles, it is difficult to obtain true aliquots for comparative studies. We therefore decided to develop a technique for the preparation of hormone-responsive thyroid cell suspensions, which is described in this paper. Its efficiency is documented by the analysis of various forms of thyroid tumours.

Some of these results have been reported in preliminary form (Breustedt et al. 1975; Nolde et al. 1975).

**Material and Methods**

*Preparation of cell suspension.* – Thyroid tissue removed by surgery was immediately put into cold buffered Earle's salt solution (Flow Laboratories, Nr. SF-041D, without Ca++, + 20 mM Hepes pH 7.4), freed from fat and connective tissue and cut into small pieces. The chopped tissue was washed four times with 5–10 vols. of buffer by slow stirring for 8 min at 4°C and removing the supernatant by suction. The tissue was finally sedimented (580 x g, 8 min), weighed and incubated in 6 vols. of the above mentioned Hepes-salt buffer containing 0.25% collagenase (Worthington, Freehold; CLS 45K148), 0.1% hyaluronidase (E. Merck; Nr. 4508) (cf. Vale et al. 1972) and 3% inactivated calf serum (GIBCo, USA). During incubation at 37°C the mixture was slowly stirred (120 r. p. m.) and every 10 min the mixture was sucked 6 times into a
70 ml syringe provided with silicone tubing (inner diameter 5 mm). After 60 min the tissue fragments were allowed to settle and the cloudy supernatant was sucked off and discarded. The residue was washed once with salt buffer and the pellet suspended in 6 vols. of warmed (37°C) salt buffer containing 0.25% Viocase (GIBCo, USA) followed by an incubation for 10–15 min at 37°C with slow stirring (120 r.p.m.). At times 10 min and 15 min of incubation the mixture was passed through a 70 ml syringe as described above and finally poured through a plastic sieve (1 mm²) into a polycarbonate tube containing inactivated bovine serum to give a final serum concentration of 10%. The cells were sedimented by centrifugation (8 min, 2000 r.p.m., 4°C) and washed 3 times in cold MEM medium containing 20 mM Hepes pH 7.4 (Flow Laboratories, Nr. 1F-121C) and 3% inactivated serum. The cells were finally re-suspended in 3–5 ml of the same medium, filtered through four layers of perlon stocking and diluted to a concentration of 1 x 10⁷ cells/ml.

Cell number was determined by counting in a haemocytometer.

Incubation of cells with TSH and determination of cAMP levels. – Aliquots of the cell suspension (0.5–1 x 10⁶ cells) were routinely incubated in Hepes-buffered MEM pH 7.2 (37°C) for 20 min ± 2 mm theophylline. Then, 200 mU/ml of bovine TSH was usually added where indicated to a final volume of 200 µl (shaking water bath, 37°C). The reaction was stopped by the addition of 50 µl 25% cold trichloroacetic acid. After centrifugation, 200 µl of the supernatant was extracted five times with 1 ml cold water-saturated ether. Aliquots were analyzed for cAMP by the method of Gilman (1970), or Brown et al. (1972), or by a modification (Schumacher, unpublished experiments) of the radioimmunoassay (RIA) (Steiner et al. 1969). All methods gave identical values.

Morphological analyses. – Before disaggregation, parts of the tumour tissues were fixed in 5% phosphate buffered formaldehyde for routine histological examination. The morphological integrity of the dispersed cells was analyzed by phase contrast microscopy. Dye exclusion tests were routinely performed in aliquots of the cell suspension by staining with 5% trypan blue in phosphate buffered saline.

For electron microscopy cells were sedimented at 1200 g for 7 min and the pellets fixed at 4°C for 90 min in 2.3% glutaraldehyde in 0.2 M cacodylate buffer pH 7.4. The fixative was removed and the pellet rinsed for 24 h at 4°C with cacodylate buffer. Post-fixation was performed for 1 h at 0°C in 2% osmium tetroxide in phosphate buffer followed by dehydration in cold alcohol. The pellets were then embedded in Epon 800. Semi-thin sections were cut with a Reichert OmU 2 microtome and the sections stained with toluidine blue for light microscopic examination. Ultra-thin sections were obtained from light microscopically selected cell clusters. The sections were counterstained in lead hydroxide solution. Electron micrographs were obtained with a Siemens EM 300 microscope.

Clinical diagnosis was based in most cases on the analysis of iodide uptake, T₃/T₄ levels in serum, TSH determination, and scintiscans.

DNA was determined according to Burton (1968).

Human TSH was kindly supplied by the hormone distribution officer (NIH) and the WHO Intern. Laboratory for Biological Standards (London), which also supplied a bovine TSH standard. Most experiments were performed with commercial bovine TSH (Thyrostimulin®, Organon GmbH, München), which exhibited activities similar to the WHO standard preparation.
Other hormones. – Prostaglandin El was a generous gift of Dr. Borgström (Lund). ACTH was obtained from Hoechst AG (Frankfurt), Glucagon from E. Lilly GmbH (Giessen), HCG from Organon GmbH (München), and HGH from Kabi AG (München).

cAMP was obtained from Boehringer (Mannheim), [3H]cAMP from Amersham-Buchler (Braunschweig).

RESULTS

1. Preparation of hormone-responsive thyroid cell suspensions

Thyroid tissue obtained at surgery was subjected to a collagenase-hyaluronidase-viocase-aided desintegration yielding cell suspensions with apparent morphological integrity as indicated by intact structures observed in light and electron microphotographs (Fig. 1), and by a positive dye exclusion test in more than 90% of the cells (not shown). Viability of the cells is also documented by the remarkable stability of basal cAMP during incubation (cf. Fig. 3) and the absence of significant amounts of cAMP in the medium even in the presence of phosphodiesterase inhibitors (not shown). Functional integrity and specificity of the cell suspensions were also indicated by their ability to respond immediately to TSH with a rapid increase in cAMP concentrations (cf. Fig. 4). Specificity of the hormonal response as documented in Table 1 is similar to

Fig. 1.
Isolated thyrocytes obtained from an adenomatous goitre. Thyroid tissue was removed at surgery and processed as described under 'preparation of cell suspension'. May-Grünwald-Giemsa stain. A = magnification 630 x; B = magnification 1900 x.
Dependence of cAMP accumulation on human and bovine TSH concentrations. Thyrocytes from an adenomatous goitre were pre-incubated with 2 mM theophylline for 20 min. TSH was added at the indicated concentrations and further incubated for 12 min. cAMP determinations were performed by RIA. Each point represents the mean of duplicate determinations. •—• human TSH; ○—○ bovine TSH (WHO standard); ■—■ bovine TSH (Organon).

thyrocytes from other species (cf. Field 1975). Besides TSH only PGE₁ led to a significant elevation of cAMP levels, which has also been noted in non-human thyroid tissue (Sato et al. 1972; Mashiter & Field 1974) and in thyroid slices from patients with Graves' disease (Takasu et al. 1976). On the other hand, cells obtained from a thyroid carcinoma later identified as a medullary (C-cell) carcinoma did not react to TSH administration by an increase in cAMP.

The thyrocytes responded to homologous and heterologous TSH in a dose-dependent manner (Fig. 2). Half-maximal stimulation was obtained with 0.4 mU/ml human TSH (about $3 \times 10^{-10}$ M), while bovine TSH proved to be 3.5–8 times less efficient. The superiority of human TSH has been verified in three different thyrocyte suspensions by determination of half-maximal concentrations, indicating significant species specificity of human TSH receptors ($P < 0.01$). When theophylline was omitted from the incubation mixture, the
maximal cAMP response was drastically reduced (not shown). No explanation can be given for the decrease in cAMP response at high hormone concentrations with these thyrocytes. In cell suspensions from other tumours, no such decrease was seen at TSH concentrations as high as 2000 mU/ml.

cAMP concentrations of thyrocytes were not significantly influenced by contaminating blood, as determined by comparative analyses of whole blood. It should also be pointed out, that only negligible amounts of cAMP were found extracellularly, whether cells were stimulated by TSH or not.

2. Kinetic analysis of TSH action on intracellular cAMP levels

When cell suspensions from various thyroid tumours were analyzed for TSH mediated increase in cAMP, remarkable differences in the time course of the response were seen (cf. Figs. 3 and 4). In most adenomatous thyroid tissues kinetics like the one in Fig. 3 B or in Fig. 4 were observed, in which most of the increase in cAMP occurred within 5 min after TSH administration. In other cases, however, a slower response was seen (Fig. 3 C), or the hormone induced change in cAMP was only transient showing return to nearly basal levels already...
Effect of theophylline on basal and TSH-induced cAMP levels. $5 \times 10^5$ thyrocytes from a non-functioning follicular adenoma were pre-incubated for 20 min and additions were then made as indicated ($\pm 2$ mM theophylline; $\pm 200$ mU/ml TSH). cAMP was determined in the RIA test as described in Methods. Each point represents the mean of triplicated determinations, with SEM values not exceeding 10%.

within 10 min after TSH addition (Fig. 3 A). This demonstrates that only kinetic analyses of the cAMP response can provide an adequate picture of the TSH action in tumour tissues.

3. **Synergistic effects of theophylline and TSH in cell suspensions from various thyroid tumours**

The tumour cell suspensions usually exhibited certain differences in basal cAMP levels as well as in the degree of response to saturating doses of TSH, although most values for basal cAMP were in the range of 2–4.5 pmoles cAMP/10⁶ cells (Table 2). Only in one case was normal thyroid tissue available, which had a basal cAMP level similar to most adenomatous goitres.

Stimulation of thyrocytes by TSH alone brought about only moderate elevations of cAMP levels in most cell suspensions obtained from adenomatous tissue as well as in the normal tissue. Comparison of five adenomatous goitres without hyperplasia or clinical dysfunction showed almost identical maximal stimulation factors of 1.75 ± 0.13 over the basal cAMP values. Theophylline alone had also
Table 1

Effects of various hormones on cAMP formation in cell suspensions obtained from a non-functioning adenomatous goitre.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>cAMP content (pmole/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>TSH 0.36 μM</td>
<td>2.97 ± 0.013</td>
</tr>
<tr>
<td>PGE₁ 28 μM</td>
<td>0.65 ± 0.003</td>
</tr>
<tr>
<td>ACTH 0.1 mM</td>
<td>0.12 ± 0.003</td>
</tr>
<tr>
<td>Glucagon 0.1 mM</td>
<td>0.14 ± 0.001</td>
</tr>
<tr>
<td>HCG 0.1 mM</td>
<td>0.14 ± 0.003</td>
</tr>
<tr>
<td>HGH 0.1 mM</td>
<td>0.13 ± 0.003</td>
</tr>
</tbody>
</table>

3.4 × 10⁶ thyrocytes were pre-incubated for 20 min at 37°C in the presence of 2 mM theophylline, and further incubated for 10 min after addition of the hormones. Details of cAMP (Gilman 1970) and DNA determinations are described in Methods. The values indicate the range of duplicate analyses.

Table 2.

Effect of TSH and theophylline on basal cAMP levels in cell suspensions from various thyroid tumours.

<table>
<thead>
<tr>
<th>Thyrocytes obtained from</th>
<th>Basal cAMP (pmole/10⁶ cells ± SEM)</th>
<th>Stimulation factor (unstimulated = 1.0; ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TSH</td>
</tr>
<tr>
<td>Normal thyroid (n = 1)</td>
<td>4.38 ± 0.16</td>
<td>1.70</td>
</tr>
<tr>
<td>Adenomatous goitre (n = 5)</td>
<td>4.10 ± 0.31</td>
<td>1.75 ± 0.13</td>
</tr>
<tr>
<td>Adenomatous goitre with hyperplasia (n = 3)</td>
<td>2.17 ± 0.16</td>
<td>3.77 ± 0.44</td>
</tr>
<tr>
<td>Hyperfunctioning adenoma (n = 2)</td>
<td>0.54 ± 0.16</td>
<td>1.20 ± 0.15</td>
</tr>
</tbody>
</table>

Isolated tumour thyrocytes were pre-incubated for 20 min at 37°C ± 2 mM theophylline as described in Methods followed by an incubation for 10 min with 200 mU/ml bovine TSH. cAMP values were determined in duplicate or triplicate in each experiment giving SEM values not greater than 10%. In Table 2, SEM values for different preparations from different patients are listed.
relatively little effect on cAMP levels. However, when combined with TSH, a more than additive effect was seen in all cases except in a hyperfunctioning adenoma (Table 2).

The inability of saturating concentrations of TSH to elevate basal cAMP levels above a factor of two in all cases of adenomatous goitre might indicate that a highly effective phosphodiesterase interfered with the accumulation of cAMP by the activated adenylate cyclase. When a kinetic analysis of the TSH effect was performed in several adenomatous goitres, TSH increased cAMP again only moderately, the elevated level remaining practically constant for at least 60 min (Fig. 4). This limitation of cAMP accumulation appeared to be due to phosphodiesterase action since addition of the inhibitor theophylline (2 mM) immediately led to a dramatic rise in the cyclic nucleotide, exhibiting a maximum at 30 min (Fig. 4).

The data in Table 2 also indicate interesting differences between the various tumour groups with respect to basal cAMP as well as to TSH and TSH + theophylline responses. Three cases of adenomatous goitre with hyperplasia exhibited low levels of basal cAMP, and a much higher degree of stimulation by TSH especially when combined with theophylline. However, it should be noted that the final cAMP levels reached did not exceed the values found with maximal TSH stimulation in adenomatous goitres and in normal tissue, i.e. the higher stimulation factor is due to the lower basal cAMP levels in these cells.

Two cases of hyperfunctioning adenoma producing excess thyroid hormones surprisingly showed very low levels of basal cAMP which could not be elevated by TSH, even when combined with theophylline. The loss of TSH response however, may be a secondary event induced by the long-standing hyperthyroxaemia which is known to drastically reduce the ability of the thyroid to activate adenylate cyclase in response to TSH (Gafni et al. 1975).

DISCUSSION

Thyrocyte suspensions have been prepared previously by trypsinization of thyroid tissue (cf. Pulvertaft et al. 1959; Tong et al. 1962; Hung & Winship 1964; Rodesch & Dumont 1967). When prepared from porcine thyroid, the cells did not respond to TSH except after cultivation for 12 h (Lissitzky et al. 1973). In some cases relatively high concentrations of TSH were required for maximal stimulation (Burke et al. 1971). The preparation of human thyroid cells by a collagenase-aided tissue desintegration first reported in a preliminary publication (Breustedt et al. 1975) is similar to a method for the isolation of pituitary cells (Vale et al. 1972). The cells proved to be highly responsive to hormonal stimulation. Even in the heterologous system (bovine TSH), half-maximal accumulation of cAMP was achieved at the same, or lower, hormone concen-
trations than those reported for the half-maximal activation of adenylate cyclase or binding of TSH in homologous bovine systems (cf. Field 1975). The human thyrocytes responded to human TSH at still lower concentrations indicating significant species specificity of the hormone receptor. The values obtained in the human system for half-maximal elevation of cAMP (3 x 10^{-10} \text{ M}) correspond to the TSH concentration giving 50% of the maximum release of iodide in hog thyroid cells (Fayet 1974). The cells could also be stimulated by PGE\(_2\), though to a lesser degree than by TSH. This is similar to thyroid tissue of other species (cf. Sato et al. 1972; Mashter & Field 1974).

The advantage of analyzing isolated thyrocytes as against tissue slices is clearly documented: Different groups of adenomas can be distinguished solely by an analysis of basal and TSH-stimulated cAMP levels. Additional information came from kinetic determinations indicating the existence of at least three types of non-functioning adenomas, one with a possible deletion in the receptor-adenylate cyclase complex (Fig. 3 C) and another (Fig. 3 A) showing an initially normal response to TSH followed by a rapid return of cAMP to basal levels. The deletion in the non-functioning adenoma presented in Fig. 3 B appears to be beyond the cAMP system: Since thyroid hormone was not produced by the nodule as indicated by scintiscan, while TSH in the isolated thyrocytes evoked a normal increase in cAMP, the defect of this non-functioning adenoma appears to be later in the sequence: hormone → adenylate cyclase → cAMP → → → hormone response, in protein kinase activation for instance, or in iodination (cf. Breustedt et al. 1976).

Furthermore, the analysis of two hyperfunctioning, autonomous nodules indicated interesting lesions. Although the nodules produced excess thyroid hormone as evidenced by scintigraphy and determination of T\(_3\)/T\(_4\) levels, they did not respond to TSH \textit{in vitro} and \textit{in vivo}\(^1\). At the same time, however, basal cAMP levels were far below the control levels. Since an elevation of cAMP and the subsequent activation of protein kinases normally appears to be an essential step in the production of T\(_4\) (cf. Dumont 1971; Field 1975), the autonomy of these adenomas in producing large amounts of T\(_4\) in spite of low, subnormal cAMP levels could be explained by a deletion which would allow production of T\(_4\) without a cAMP dependent activation of protein kinases. One possible explanation would be that protein kinases of these cells are active in the absence of cAMP, presumably by a deletion in the regulatory subunit R. Such lesions have already been described for some hepatomas (cf. MacKenzie & Stellwagen 1974). The inability of these cells to respond to TSH, then, appears to be a secondary effect of the longstanding hyperthyroxinaemia, which is known to suppress the response of the adenylate cyclase to TSH (Gafni et al. 1975).

\(^1\) In order to prove TSH unresponsiveness in this tumour \textit{in vitro}, the data should also be supplemented by an analysis of different TSH concentrations.
Two other cases of 'hyperfunctioning' adenomas have previously been analyzed (Burke & Szabo 1972) with the aid of the tissue slice technique. There was no difference in basal cAMP between nodules and adjacent 'normal' tissue, but a higher response of the 'autonomous' nodules to TSH, as compared to the para-adenomatous tissue. It should be pointed out that a final elucidation of such deletions and a biochemical classification of thyroid tumours will require a systematic analysis of additional parameters like protein kinase activation, phosphodiesterase activity and thyroglobulin synthesis.

The kinetic analyses of the TSH action also provided data on hormone mediated accumulation of intracellular cAMP and the interplay of adenylate cyclase and phosphodiesterase activities in protein kinase activation. Although a phosphodiesterase present in the cells prevented the marked increase in cAMP seen when the degrading enzyme was blocked by theophylline, the enzyme was not able to degrade the cAMP fraction formed by TSH alone, which persisted over 60 min. This fraction, therefore, may be different from the bulk of cAMP produced by the stimulated adenylate cyclase, which is rapidly degraded by phosphodiesterase except when protected by theophylline. Protection against phosphodiesterase of cAMP bound to proteins like the regularly subunit R of protein kinases has been observed in homogenates and in cells (Goldberg et al. 1970; Cheung & Patrick 1974; Hilz et al. 1975). The protein-bound, phosphodiesterase-resistant cAMP fraction also behaved differently from total cAMP in the glucagon-stimulated rat liver and proved to be a direct measure of R·cAMP and the activation state of protein kinase(s). Furthermore, it exhibited a greatly retarded decay in glucagon-stimulated rat livers (Schwoch & Hilz 1977). In analogy, the cAMP fraction in TSH-stimulated thyrocytes resistant to phosphodiesterase for more than 60 min may also represent cAMP bound to the regulatory subunit of protein kinase, thus keeping the catalytic subunits active over an extended period. However, it cannot be completely excluded that the less marked rise in cAMP in the absence of theophylline is due to a new hormone-induced steady state between cAMP formation and degradation.

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ADDENDUM

During the preparation of the manuscript, two papers came to our knowledge which described the preparation, cultivation and analysis of human thyrocytes (Kaneko 1975; Bidey et al. 1976).
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