EVIDENCE FOR DIFFERENTIAL SYNTHESIS OF THYROXINE AND TRI-IODOTHYRONINE BY CULTURED HUMAN THYROID CELLS FOLLOWING EXPOSURE TO THYROTROPHIN OR DIBUTYRYL CYCLIC AMP

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SUMMARY

Cultured human thyroid cells treated with thyrotrophin (TSH) or dibutyryl cyclic AMP release more tri-iodothyronine (T3) and thyroxine (T4) than unsupplemented cells. Column chromatography was used to investigate the secretion of newly-synthesised 125-I labelled T3 and T4 from cells cultured with 125-I and TSH or dibutyryl cyclic AMP. Radioimmunoassays were used to determine total T3 and T4 release from cells cultured with unlabelled iodide.

Iodothyronines released after TSH addition contained more 125-I than those released after dibutyryl cyclic AMP. This increase in 125-I was primarily in "new" T4. Release of "new" T3, however, was increased more by dibutyryl cyclic AMP than by TSH. Dibutyryl cyclic AMP and TSH were comparable in their stimulation of total T3 and total T4 release.

Interpretation of these observations suggests that TSH and dibutyryl cyclic AMP may differ in some aspects of their in vitro effects on cellular iodination and iodothyronine coupling systems.

INTRODUCTION

Stimulation of thyroid hormone secretion by TSH has been demonstrated in a variety of in vitro studies (1 - 3). A similar response has been elicited by the addition to the culture medium of dibutyryl cyclic AMP (DBcAMP)(4,5). Either treatment has been shown to produce a net increase in intracellular cyclic AMP levels (6). One overall result of these increases in cyclic AMP is that of protein kinase activation, with subsequent phosphorylation of thyroidal enzymes into an active form (7). However, evidence has accumulated which suggests that there may be more than one thyroidal protein kinase species and that the role of cyclic AMP in activating certain of these enzyme systems is by no means a universal one (8,9). In particular, cyclic GMP has been shown to be responsible for many of the functions previously attributed to cyclic AMP, in a variety of cellular activations (10).

We report in this communication preliminary evidence which supports the theory of a differential degree of enhancement of thyroidal pathways responsible for iodothyronine synthesis, following TSH and DBcAMP respectively. In particular, the relative rates of de novo synthesis and release of T3 and T4 by isolated cultured human thyroid cells may differ as a function of the stimulus to which the cells are subjected.

METHODS

Cell culture

Normal human thyroid tissue was obtained from patients undergoing parathyroidectomy or removal of cold benign thyroid cysts. Excised tissue was washed in Hank's salt solution at 4°C, then freed of connective tissue and chopped into 1-2 mm cubes. These were then stirred with 0.25% (w/v) trypsin in Hank's solution at 37°C for 20 minutes, after which a fresh trypsin solution was substituted, and the old solution, containing red blood cells was discarded. After 90 minutes, the thyroid cell suspension was decanted and the cells pelleted at 600 x g. Residual trypsin was inactivated by suspending the cell pellets in 0.25% (w/v) soybean trypsin inhibitor in Hank's solution for 30 minutes at 37°C, followed by pelleting at 600 x g. Thyroid cell pellets were then resuspended in Eagle's Basal Medium, containing 25mM HEPES buffer, 10% (v/v) foetal calf serum, 1μM sodium iodide, and 200μg/ml gentamicin (Flow Laboratories, Irvine, Ayrshire, Scotland). A plating density of approximately 2 x 10^5 cells was obtained with a haemacytometer. Five millilitre aliquots of this cell suspension were incubated in 25cm^2 cell culture flasks at 37°C.

Assay of cells for DNA

After the in vitro growth period, cells were removed from the culture flasks by incubation with 0.06% (w/v) trypsin in Hank's solution. The pellets of cells obtained after centrifugation were resuspended in 2ml of 0.05M sodium phosphate buffer, pH 7.4 at 4°C, and the suspensions freeze-thawed twice in an acetone - solid CO_2 mixture. The DNA content of duplicate 500μl aliquots of each cell lysate was determined by the Method of Leyva and Kelley (11).

Effects of thyrotrophin and dibutyryl cyclic AMP

Bovine TSH (Sigma London Chemical Company, Kingston, Surrey, U.K.) was dissolved in 1ml of sterile distilled water containing 3mg of phenol and 50mg of glucose. Cultures were supplemented with this sterile solution at the time of cell plating, to give a final concentration of 100mU/ml.

Dibutyryl cyclic AMP (Boehringer Mannheim) was dissolved in 0.05M sodium phosphate buffer, pH 7.4 to give a final concentration of 100mM. This stock solution was sterilised by membrane filtration and used to supplement cell cultures to a final concentration of 1.0mM.

Thyroxine radioimmunoassay

The release of T4 by cultured cells was estimated by a modification (12) of a standard T4 radioimmunoassay (13), and in addition, the T4 standards were double-diluted in sodium barbitone buffer (0.08mol/l.) containing an appropriate dilution of fresh culture medium instead of human serum. Aliquots of culture medium were withdrawn at 24, 48 and 140 hours, and routinely diluted 1 + 3 in culture medium so that the final percentage bound fraction of the hormone fell on the steep portion of the standard curve.

Tri-iodothyronine radioimmunoassay

The levels of T3 in aliquots of withdrawn culture medium were determined by a modified (14) standard T3 radioimmunoassay (12), with the additional modifications described for the T4 radioimmunoassay.

Estimation of newly synthesised T3 and T4 in culture medium

Cultures which were to be used for the estimation of newly-synthesised iodothyronines (T3 and T4) were supplemented with [125-I] sodium iodide (carrier free, the Radiochemical Centre, Amersham, Bucks, U.K.) at the

Table 1

Effect of TSH and DBcAMP on total and newly synthesised T3 released from human thyroid cell cultures after 24, 48 and 140 hours. ND = none detected.

Treatment	time (h)	total T3 ng/μg DNA (Mean±SEM, n = 6).	new T3 125-I cpm/μg DNA (Mean±SEM, n = 6).	cpm/ng
TSH 100mU/ml	24	0.95 ± 0.4	156 ± 9.7	164
	48	0.7 ± 0.2	ND	-
	140	0.5 ± 0.35	ND	-
DBcAMP 1.0mM	24	0.74 ± 0.2	333 ± 28.7	450
	48	0.8 ± 0.17	84 ± 17.2	105
	140	0.7 ± 0.3	42 ± 6.4	60
Controls	24	0.5 ± 0.05	61 ± 11.2	122
	48	0.47 ± 0.05	31 ± 9.6	66
	140	0.25 ± 0.15	15 ± 5.3	62

time of cell plating to produce a final activity of 1.0μCi/ml. Aliquots of medium withdrawn at 24, 48 and 140 hours were fractionated on columns of Sephadex G-10 (Pharmacia, Uppsala, Sweden) using a two stage fractionation procedure (15). In the first stage, the eluant was 0.5M TRIS-maleic acid-NaOH, pH6.9, and in the second stage, 0.05M TRIS-HCl, pH9. Column dimensions were 0.9 x 15cm and buffer was eluted at a rate of 10ml/hour. Eight minute fractions were collected and counted on a Packard Autogamma counter.

RESULTS AND DISCUSSION

The principal objective of these studies was to determine the relationship between "new" and total T3 and T4 secretion following differential exposure of normal human thyroid cell cultures to TSH and DBcAMP. The T3 and T4 detected by radioimmunoassay will each be composed of two components, i.e. stored hormone, released in response to agents stimulating secretion, and "new" hormone, which under conditions of stimulation may be secreted immediately after formation. In this case, storage as thyroglobulin bound hormone would be minimal. In order to study the secretion of newly-iodinated iodothyronines and the extent to which this process might be differentially influenced by TSH or DBcAMP, groups of cultures were supplemented with ^{125}I in addition to unlabelled sodium iodide. Medium withdrawn from these cultures was fractionated on Sephadex G-10, and the ^{125}I activity associated with each of the iodothyronine peaks was measured.

Table 1 compares the extent of "new" T3 synthesis with the total

Table 2

Effect of TSH and DBcAMP on total and newly synthesised T4 released from human thyroid cell cultures after 24, 48 and 140 hours.

Treatment	time (h)	total T4 ng/µg DNA (Mean±SEM, n = 6).	new T4 125-I cpm/µg DNA (Mean±SEM, n = 6).	cpm/ng
TSH 100mU/ml	24	10.5 ± 0.45	880 ± 72	84
	48	9.1 ± 1.5	265 ± 24.6	29
	140	4.1 ± 0.8	125 ± 17.4	30
DBcAMP 1.0mM	24	10.2 ± 2.0	42 ± 2.4	4
	48	5.6 ± 2.3	20 ± 2.0	4
	140	4.5 ± 1.7	13 ± 1.4	3
Controls	24	4.75± 0.75	138 ± 14.1	27
	48	4.5 ± 1.02	62 ± 8.1	14
	140	1.6 ± 0.5	75 ± 8.3	47

amount of T3 released, after TSH or DBcAMP. T3 levels in both TSH and DBcAMP supplemented cultures were elevated to a comparable extent after 24 hours, but the T3 released from cells exposed to DBcAMP showed a significant ($p < 0.05$) increase in specific activity of ^{125}I over that observed in TSH supplemented cultures. Although the level of newly-iodinated T3 was thereafter diminished in DBcAMP supplemented cultures (Table 1), no ^{125}I-T3 was detectable in TSH supplemented cultures after this time, although comparable levels of unlabelled T3 were still observed.

Table 2 gives data obtained for "new" T4 synthesis, in relation to total T4 release, after TSH or DBcAMP. Total T4 levels detected by radioimmunoassay at 24 hours were elevated to a comparable extent by TSH or DBcAMP above those found in control cultures. TSH significantly ($p < 0.001$) increased the synthesis of ^{125}I-T4 above the level observed in control cultures, although incorporation of ^{125}I into T4 by DBcAMP supplemented cells was minimal, and significantly ($p < 0.001$) less than that shown by control cells. In direct contrast was the effect of DBcAMP in increasing the levels of "new" ^{125}I-T3.

Incorporation of ^{125}I into both T3 and T4 in unsupplemented cells suggested a residual capacity of the cells for limited iodothyronine synthesis, although some exchange of free ^{125}I for residual iodide stored on thyroglobulin cannot be discounted.

Two main conclusions may be drawn from the findings expressed in Tables 1 and 2. Firstly, no significant difference in total T3 or T4 release was observed between DBcAMP and TSH treated cultures, although either treatment increased the levels of both T3 and T4 above those observed in control cultures. Secondly, a clear differential effect on the incorporation of ^{125}I into T3 and T4 was apparent, depending on whether the cells were exposed to TSH or DBcAMP. Thus ^{125}I-T4 levels were minimal following DBcAMP whilst ^{125}I-T3 levels were increased. After TSH, ^{125}I-T4 was significantly ($p < 0.001$) increased at the eventual expense of ^{125}I-T3, although initial ^{125}I-T3 levels were still greater than those in control cultures.
It should be mentioned that the progressively decreased levels of T3 and T4 observed in all cultures with time probably resulted from a post-secretory deiodination to mono- and di-iodotyrosine (MIT and DIT). This was most likely due to cellular deiodinase leakage, since the lack of a basement cell membrane under in vitro conditions (16) may potentiate this loss. Moreover, no deiodination of T3 or T4 has been observed in preliminary experiments with cell-free culture medium, which rules out the possibility of action of serum deiodinases within the culture medium. Hence an apparently unchanged level of T4 between sampling intervals reflects an absolute secretion rate equal to that of deiodination, whilst any increased rate of hormone secretion or deiodination will increase or decrease, respectively, the apparent hormone level.

In view of the differing sensitivity of thyroidal protein phosphokinases to cyclic AMP (8,9) and the recent findings of prostaglandins in the thyroid (17), some of which are increased by TSH but not by cAMP, it would appear not unlikely that certain thyroidal enzyme systems might specifically require other TSH enhanced intermediates apart from cAMP (or DBcAMP) for activation. In the present studies, both TSH and DBcAMP appeared equipotent in their stimulation of iodothyronine release, in agreement with the findings made by earlier workers (4). However, the clear difference in the incorporation of ^{125}I into iodothyronines, in response to TSH or DBcAMP suggests that certain thyroidal enzyme systems may specifically require TSH for activation. Since T4 synthesis involves the linkage of two molecules of DIT, any diminished iodination of MIT to DIT would allow relatively more T3 to be synthesised, (MIT + DIT), as found in our experiments using DBcAMP, whilst the lack of DIT would diminish T4 synthesis. This offers an explanation as to why the specific ^{125}I activity in T3 was higher in DBcAMP than in TSH supplemented cultures. In the presence of TSH, additional factors may become available, allowing the inhibition of DIT synthesis to be reversed, with a corresponding increased synthesis of

^{125}I-T4, and a decrease in the release of ^{125}I-T3. This hypothesis is supported by the figures in Table 2, which clearly show a higher specific ^{125}I-T4 activity after TSH than after DBcAMP.

In summary, the observed differences in the specific activities of ^{125}I released as T3 and T4 following DBcAMP and TSH respectively, are in accord with the view that TSH stimulates the production of intermediary factors other than cAMP which, in turn, activate enzymic pathways promoting T4 synthesis, and that in the absence of these factors, T3 synthesis predominates. In addition, the release of similar levels of total iodothyronines suggests that DBcAMP and TSH may have similar effects on iodothyronine secretion mechanisms, and also that release of newly-synthesised T3 and T4 accounts for only a small proportion of total release.

The precise identities and roles of cellular intermediates other than cAMP are not yet known with any certainty. Nevertheless, the recent findings of cAMP-dependent and cAMP-independent protein kinases within the thyroid follicular cell (9) and also prostaglandins specifically requiring TSH for their activity (17) do suggest that iodothyronine synthesis is regulated by complex systems, although secretion may depend on different mechanisms. Moreover, since recent work (10) has shown cyclic GMP to control many of the metabolic pathways hitherto assigned to cyclic AMP, this molecule would appear to be a likely candidate for investigation within the thyroid cell.

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