A role for intracellular calcium and calmodulin in the release of triiodothyronine from human thyroid-cell monolayer cultures

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Human thyroid cells in monolayer responded to acute stimulation by TSH with an increase in the secretion of T3. This process appeared to be dependent on a rise in the cytosolic calcium concentration since the antagonist of intracellular calcium mobilization, TMB-8, was found to inhibit the release of T3 in response to TSH. The importance of intracellular calcium was further shown using the agent veratridine which increases the free calcium level within cells; veratridine potentiated the stimulation of T3 secretion by TSH and itself stimulated the release of T3 to a level higher than that seen in the presence of TSH alone. The calcium ionophore A23187 produced a biphasic effect on T3 secretion from human thyroid monolayers; at low concentrations, A23187 caused a decrease in both unstimulated and TSH-stimulated T3 secretion but above a concentration of 1 μM, T3 secretion was increased. The calmodulin antagonist W7 was found to inhibit T3 release in response to TSH, indicating a role for calmodulin in mediating the effects of intracellular calcium on T3 secretion.

The thyroid gland provides an example of a cellular system where calcium ions and cyclic nucleotides act as dual interrelated messengers in controlling thyroid responses to the pituitary hormone thyrotrophin (TSH), the major physiological regulator of the thyroid in vivo. The primary action of TSH in increasing intracellular adenosine 3'5' cyclic monophosphate (cyclic AMP) levels through interaction with the adenylate cyclase system is now well established (Dumont, 1971). Cyclic AMP then initiates mechanisms involved in the secretory response to TSH. Over recent years the importance of calcium in the thyroid has been increasingly recognized. However the relationship between the two intracellular messengers in the regulation of thyroid hormone secretion has yet to be established. Thyroid hormone secretion is not dependent on calcium in the extracellular fluid (Willems et al., 1971), but through a cyclic-AMP-dependent process TSH has been shown to increase the release of diffusible calcium from thyroid slices (Rodesch et al., 1974). This could initially correspond to the release of calcium from an intracellular binding site into the
cytosol (Rodesch et al., 1976). Consequently the effect of TSH in increasing cyclic AMP levels could lead to an increase in the intracellular calcium concentration allowing possible interactions between these messengers in mediating the thyroid-cell responses to TSH stimulation.

It has become clear that many calcium-dependent events within a cell are mediated by its binding to and activation of an intracellular calcium-binding protein, calmodulin. We have isolated and purified calmodulin from the human thyroid and demonstrated, using calmodulin antagonists, a possible role for calmodulin in the control of cyclic AMP production in response to TSH (Ollis et al., 1983). Furthermore we have recently obtained indirect evidence that human thyroid adenylate cyclase activity is regulated by calmodulin (Mac Neil et al., 1983).

This study investigates the role of intracellular calcium and calmodulin in the secretion of triiodothyronine (T$_3$) from monolayers of human thyroid cells, a TSH-sensitive process which we have recently described (Ollis et al., 1984). We used agents known to affect the actions of intracellular calcium and calmodulin: 8-(N,N-diethylamine)-octyl-3,4,5-trimethoxybenzoate (TMB-8), an inhibitor of intracellular calcium mobilization (Malagodi & Chiou, 1974; Charo et al., 1976; Gorman et al., 1979); veratridine, a mobilizer of intracellular stores of calcium through an action of increasing cytosolic sodium (Richardson et al., 1980); A23187, a calcium ionophore which also has an intracellular site of action in releasing calcium stores (Feinman & Detwiler, 1974; Babcock et al., 1976; Pickett et al., 1977; Thomas et al., 1981); and N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W7), an inhibitor of calmodulin activity (Hidaka et al., 1978).

**Materials and Methods**

A23187 was obtained from Calbiochem (Behring) and TMB-8 from Aldwych Chemical Company (Dorset). Medium 199, phosphate-buffered saline (PBS), foetal calf serum, L-glutamine, penicillin and streptomycin, fungizone, and trypsin were all obtained from Gibco-Europe Ltd. (Paisley). Bovine serum albumin (fraction V powder) was obtained from Armour Pharmaceutical Company (Eastbourne) and TSH (bovine pituitary, 1 unit/mg) from Sigma (London). $^{125}$I-triiodothyronine (>200 Ci/mM) was obtained from Amersham International (Bucks). All other chemicals were of analytical grade. All tissue-culture plastics were obtained from Sterilin (Teddington). W7 and veratridine were gifts from Dr. M. G. M. Blackburn, Sheffield University, and Professor E. Flückiger, Sandoz (Basle), respectively.

Cultured human thyroid cells were obtained from post-operative samples of human thyroid glands and maintained in culture under standard conditions as previously described (Ollis et al., 1982). For incubation with test agents cells were passaged into 6-well dishes and at confluence were incubated during a 16-h incubation period in the absence or presence of TSH (10 mU/ml) as reported (Ollis et al., 1984) with the following drugs: TMB-8, veratridine, A23187, and W7. At the end of the incubation period the supernatant medium was removed and stored at -20°C for radioimmunoassay of T$_3$ released.
Cell protein was determined by the method of Lowry (Lowry et al., 1951) and results are expressed as pmol of T₃ released/mg of cell protein. Each experiment was performed at least three times and data from one representative experiment are shown.

TMB-8, veratridine, and W7 were all soluble in aqueous medium. A23187 was made up in dimethylsulphoxide (DMSO) and added to the cultures to give a final DMSO concentration of not greater than 1% (v/v), a concentration found to have no effect on unstimulated and TSH-stimulated T₃ release.

Results

The secretion of T₃ from monolayer cultures of human thyroid cells could be stimulated by TSH in a concentration-dependent manner. T₃ was detected in the incubation medium in response to a TSH concentration of 50 μU/ml and release was maximally stimulated at 10

![Graph](image-url)  
**Fig. 1.** Effect of TMB-8 on unstimulated secretion (x) and TSH-(10 mU/ml)stimulated secretion of T₃ (●) from cultured human thyroid cells during a 16-h incubation period. Each point is the mean ± S.E.M. of T₃ in triplicate cultures.
mU/ml. This maximally stimulating concentration of TSH was used in all following experiments.

The effects of the inhibitor of intracellular calcium mobilization, TMB-8, were investigated over the TMB-8 concentration range 0.1 to 100 μM on both unstimulated and TSH-stimulated T₃ release (Fig. 1). TSH-stimulated T₃ release was inhibited to a greater extent than T₃ released under unstimulated conditions (68 ± 12% compared with 53 ± 8%, (n = 3)). Maximal inhibition was seen at a TMB-8 concentration of 100 μM which decreased TSH-stimulated T₃ release to the unstimulated level.

The effects of increasing the intracellular calcium concentration were then investigated using the agent veratridine (Fig. 2). At concentrations above 50 μM, veratridine was found to increase the release of T₃ from human thyroid cells; not only did it potentiate TSH-stimulated T₃ release but it stimulated T₃ secretion itself to a level higher than that seen in the presence of TSH alone. At a

![Fig. 2. Effect of veratridine on unstimulated secretion (x) and TSH-(10 mU/ml)stimulated secretion of T₃ (●) from cultured human thyroid cells during a 16-h incubation period. Each point is the mean ± S.E.M. of T₃ in triplicate cultures.](image-url)
maximal veratridine concentration of 500 μM, TSH-stimulated and unstimulated T<sub>3</sub> release had both increased to the same level.

Fig. 3 shows the effects of the calcium ionophore A23187 on T<sub>3</sub> secretion from cultured human thyroid cells: at low A23187 concentrations there was a decrease in unstimulated and TSH-stimulated T<sub>3</sub> secretion to approx. 50% of that released in the absence of A23187. Above a concentration of 0.1 μM both unstimulated and TSH-stimulated T<sub>3</sub> release were increased; at 5 μM A23187, T<sub>3</sub> secretion was increased by 50% of that secreted in the absence of A23187. Concentrations higher than 5 μM proved toxic to the thyroid cells as assessed by cell viability.

Using the naphthalene sulphonamide W7, a calmodulin inhibitor, a possible role for calmodulin in TSH-stimulated T<sub>3</sub> release was shown (Fig. 4). In the presence of W7, TSH stimulation of T<sub>3</sub> secretion was inhibited in a concentration-dependent manner. TSH-stimulated T<sub>3</sub> release was inhibited to a greater extent than unstimulated T<sub>3</sub> release. At 100 μM W7, T<sub>3</sub> secretion in response to a maximally stimulating concentration of TSH was decreased to the level of T<sub>3</sub> released under unstimulated conditions. This corresponded to an inhibition of TSH-stimulated T<sub>3</sub> release of 75% compared to 50% inhibition of unstimulated release by W7.

![Graph showing the effect of A23187 on T<sub>3</sub> secretion](image-url)

Fig. 3. Effect of A23187 on unstimulated secretion (x) and TSH-(10 mU/ml) stimulated secretion of T<sub>3</sub> (●) from cultured human thyroid cells during a 16-h incubation period. Each point is the mean ± S.E.M. of T<sub>3</sub> in triplicate cultures.
Fig. 4. Effect of W7 on unstimulated secretion (x) and TSH-(10 mU/ml) stimulated secretion of T₃ (●) from cultured human thyroid cells during a 16-h incubation period. Each point is the mean ± S.E.M. of T₃ in triplicate cultures.

Discussion

Human thyroid cells in monolayer were responsive to TSH stimulation under established experimental conditions (Ollis et al., 1984) as measured by the secretion of T₃. The importance of mobilization of calcium from intracellular binding sites in this process was demonstrated by the use of agents to decrease or increase calcium release. TMB-8, which inhibits calcium mobilization, was found to inhibit TSH-stimulated T₃ release with a lesser reduction in unstimulated T₃ release. Veratridine acts by increasing cytosolic sodium which displaces bound calcium thus leading to an elevated cytosolic calcium. It was found to potentiate TSH-stimulated T₃ secretion with a concentration-dependent effect on the unstimulated release of T₃. The
calcium ionophore A23187 had a biphasic effect on both TSH-stimulated and unstimulated T₃ secretion; low concentrations had an inhibitory effect on T₃ release and a stimulatory effect at higher concentrations. Studies with the calmodulin antagonist W7 suggested that calmodulin may have a role in mediating the secretory response to intracellular calcium.

The thyroid is unusual amongst secretory glands in that it does not require extracellular calcium for thyroid hormone secretion when stimulated by TSH (Willems et al., 1971) and in fact extracellular calcium depletion has been reported to stimulate the release of thyroxine (T₄) from mouse thyroids (Maayan et al., 1981). However an important role for intracellular calcium in the thyroid is recognized. TSH is known to increase the release of diffusible calcium from thyroid slices (Rodesch et al., 1976), the source for an increased calcium efflux being an intracellular compartment of the thyroid cell (Rodesch et al., 1976). Consequently TSH stimulation may be associated with an increase in the free cellular calcium concentration which, however transient, could initiate a series of calcium-dependent processes involved in thyroid hormone secretion allowing interactions between calcium and cyclic AMP in thyroid cell regulation. One possible site of interaction could be the production of cyclic AMP by the membrane-bound enzyme adenylate cyclase, whose activity is known to be inhibited by high concentrations of calcium and stimulated by low concentrations in the presence of the calcium-chelating agent EGTA (Desmedt & Dumont, 1977).

The inhibitory effect of high concentrations of calcium on adenylate cyclase may explain in part the depressive action of the calcium ionophore A23187 on unstimulated and TSH-stimulated T₃ release. Agents which increase the influx of calcium in the thyroid have been found to increase cyclic GMP, activate protein iodination and [1-¹⁴C]glucose oxidation, and depress cyclic AMP accumulation and secretion in dog thyroid slices (Van Sande et al., 1975). This was a direct effect of calcium on these cellular processes as the inhibitory effect of A23187 on cyclic AMP was suppressed in calcium-depleted slices (Van Sande et al., 1979). At higher concentrations of A23187 the increase in T₃ secretion may be due to the other intracellular actions of calcium involved in processes associated with hormone secretion.

The role of calmodulin in mediating many calcium-dependent cellular responses is well recognized (Tomlinson et al., 1984). Calmodulin is thought to have a possible role in the responses of thyroid cells to TSH with an effect of calmodulin inhibitors on cellular accumulation of cyclic AMP in response to TSH (Ollis et al., 1983). It is possible that the inhibitory effect of W7 on T₃ secretion described in this study may be due in part to an effect of calmodulin inhibition directly on the adenylate-cyclase/phosphodiesterase system. Calmodulin-sensitive phosphodiesterase has been described in dog thyroid (Miot et al., 1983) and the regulation of thyroid-membrane adenylate cyclase by calmodulin reported (Mac Neil et al., 1983). The involvement of calmodulin in processes after cyclic AMP formation and degradation should be considered; calmodulin has been implicated in microtubule polymerization and microfilament function (Marcum et al., 1978), processes which could be involved in lysosome fusion with colloid droplets in thyroid cytosol.
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References