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Original Article

The Inhibitory Effect of Iodide on TSH-stimulated Growth of Cultured Rat Thyroid Cells (FRTL-5)*

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Abstract: We studied the inhibitory effect of iodide on thyroid growth in FRTL-5 cells, which preserve many thyroid functions. When the amount of iodide in medium exceeds 10^{-6} M, TSH-stimulated [^{3}H]thymidine incorporation into DNA was significantly inhibited. Methimazole (0.5 mM) reversed the suppression, as was observed in other functions such as thyroid hormone synthesis and secretion. Cell numbers were also monitored to ensure that [^{3}H]thymidine incorporation reflected cell multiplication. Iodide inhibited TSH-stimulated cAMP formation and also 8-bromo-cAMP stimulated-[^{3}H]thymidine incorporation into FRTL-5 cells, indicating that it acts on steps both before and after the formation of cAMP. The data demonstrated an autoregulatory role of iodide on the growth of FRTL-5 cells.

Key words: FRTL-5, iodide metabolism, thymidine incorporation, thyroid, cAMP

It is generally accepted that most thyroid functions, including thyroid cell growth, are controlled by TSH1,13). It is also well known that excess iodide appears to depress the stimulatory action of TSH on cAMP accumulation in thyroid tissues9,18,19). Even more intriguing is the effect of iodide on thyroid growth. In dog thyroid cells of primary culture, the effect of iodide on thyroid growth is mediated, at least in part, by regulation of the thyroid amino acid transport system7). In this study, we examined the inhibitory effect of iodide on TSH-stimulated growth of FRTL-5 cells, which preserve many thyroid functions.

MATERIALS AND METHODS

Materials

The following materials were used: Bovine TSH (Thytropar, 0.5 U/mg, Armour Pharmaceutical Co., Phenix, AZ.; or 0.5 U/mg, Sigma Chemical Co., St. Louis, Mo, USA); Calf serum, Ham's F12K medium (Flow Laboratories, Rockville, MD, USA); Dulbecco's modified Eagle's medium (DME) (Gibco, Grand Island, NY, USA); Insulin, transferrin (Sigma Chemical Co.); Glycyl-L-histidyl-L-lysine acetate (Aldrich Chemical Co., Inc., Millwaukee, WI, USA); Hydrocortisone (Nikken Chemical Co., Tokyo, Japan); Trypsin (Difco Laboratories, Detroit, MI, USA); Methyl-[^{3}H]thymidine, 81.9 Ci/mmol (New England Nuclear Corp., Boston, MA, USA). All other chemicals were reagents for analytical grade.

Cells

The FRTL-5 cells used in this report are a continuous line of functional epithelial cells from rat thyroid, kindly supplied by Dr. L. D. Kohn (NIH, Bethesda, MD, U.S.A.). The isolation, growth, and basic

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characteristics of cells have been described by Ambesi-Impiombato *et al.*11) The cells were grown in modified Ham's F-12K medium supplemented with 5% calf serum and 6 hormone mixture (6H) consisting of insulin (10 μg/ml), hydrocortisone (10^{-8} M), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), somatostatin (10 μg/ml), and TSH (10 mU/ml) in a 5% CO_2—95% air, 37°C humidified atmosphere, and were passaged biweekly with a split ratio of 1 : 10 using 0.25% trypsin solution containing 2 mM EGTA. For all assays, cells were seeded in 12-well tissue trays (approximately 2×10^5 cells/well) in Ham's F-12K medium containing 5% calf serum, unless stated otherwise. After 4 days, medium was aspirated, wells were washed twice with HBSS (Hank's balanced saline solution) and then incubated in Ham's F-12K containing 5% calf serum without additives (basal medium).

Growth and [^{3}H]thymidine incorporation assays

After 3 days, basal medium was aspirated. Cells were preincubated in fresh medium containing 5% calf serum with one or more additives for 6 h. Then [^{3}H]thymidine was added, and the cells were further incubated for 24 h to measure thymidine incorporation. Cells were then washed twice with ice-cold HBSS, followed by two washes with ice-cold 10% trichloroacetic acid (TCA). After aspiration of the supernatant, 300 μl of 0.2 N NaOH was added to solubilize the cells. For determination of [^{3}H]thymidine incorporation, 100 μl was neutralized with 1 N HCl, and radioactivity was counted in a liquid scintillation spectrometer. The DNA content of each dish was measured by a modification of the method of Burton3). Briefly, to the remaining 200 μl of solubilized cell solution in 0.2 N NaOH, 200 μl of 1 N perchloric acid and 800 μl of diphenylamine solution were added. This solution consisted of 1.5 g diphenylamine in 100 ml glacial acetic acid and 1.5 ml sulfuric acid with 500 μl of 16 mg/ml acetaldehyde. After development for 24 h at room temperature, aliquots of each well were analyzed for DNA content by absorbance at 600 nm using calf thymus DNA as the standard.

For determination of cell number, viable cells were directly counted in a Neubauer chamber after release from plates by the 0.25% trypsin solution.

Iodide uptake assay

We adopted the method described by Tong4), modified for FRTL-5 cells1). About 1.0×10^6 cpm of ^{125}I per dish was added to the basal medium in the presence of 1 μM cold iodide and 1 mM methimazole (MMI) to inhibit organification of iodide. Cells were incubated for 2 h, and at the end of the incubation the medium was removed and its radioactivity was determined. Cells were then washed twice with ice-cold HBSS, scraped with a rubber policeman, pelleted in pre-weighed tubes and assayed for radioactivity. The C/M ratio (i.e., cpm /μl of medium: cpm/mg of cell pellet) was calculated.

Cyclic AMP measurement

After 3 days of incubation in a basal medium (5% calf serum with no additives), the medium was removed, and the cells were incubated for 2 h in 1 ml of HBSS containing 0.5 mM 3-isobutyl-1-methylxanthine and selected concentrations of TSH. The medium was then taken, and deproteinized by heating in a boiling water bath for 10 min. Aliquots of the medium were analyzed for cAMP by RIA using Yamasa's cAMP RIA kit.

NRK cells

NRK cells (ATCC CRL 1570: normal rat kidney fibroblasts) were maintained in DME with 5% calf serum (CS) and were split in a 1 : 10 ratio when subconfluent.

Cells from subconfluent plates were distributed into 12-well plates at a density of 5×10^3 cells per well in DME with 5% CS. After 24 h, the medium was removed and iodide uptake was measured by the method noted before.

RESULTS

TSH dependent properties of FRTL-5 cells

TSH stimulated cAMP release from FRTL-5 cells into the medium in a dose-dependent manner. In the presence of 100 μU/ml TSH, cAMP release was increased 15-fold over basal levels. Maximal cAMP stimulation was obtained with 1 mU/ml TSH (Fig. 1). Iodide uptake was measured both in FRTL-5 cells and NRK cells to determine the C/M ratio. FRTL-5 cells were able to concentrate iodide at levels as much as 30 times the external concentration, while cells of nonthyroid origin (NRK) had a C/M ratio close to 1.0 (Table 1). When cells were incubated for 24 h with varying concentrations of TSH, stimulation of [^{3}H]thymidine incorporation was initially observed at a TSH concentration of 50 μU/ml while half maximal was at 250 μU/ml and maximal at 10 mU/ml (Fig. 2). Forskolin and a stable analogue of cAMP, 8-bromo-cAMP, both stimulated [^{3}H]thymidine incorporation in a dose related manner, indicating that at least a part of TSH-stimulated [^{3}H]thymidine incorporation is mediated by cAMP (Table 2).

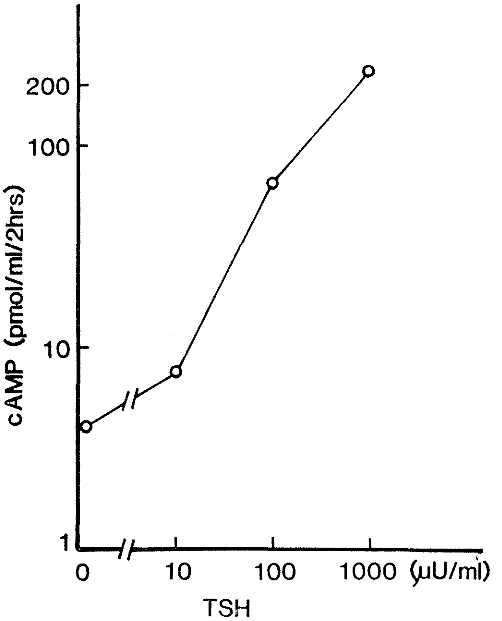

Fig. 1. Dose-response curve of TSH effect on cAMP release from FRTL-5 cells. Cyclic AMP was measured at 2 h as described in 'Materials and Methods'. Results are the average of duplicate cultures.

Table 1. Iodide uptake by FRTL-5 cells and NRK.

Cells	TSH (μU/ml)	C/M ratio*
FRTL-5	0	5.1
	100	31.0
NRK**	0	1.2
	100	1.1

$$* \ C/M=\frac{\text{cpm/mg of cell pellet}}{\text{cpm}/\mu\text{l of medium}}$$

** Rat kidney fibroblast

Details were described in 'Materials and Methods'

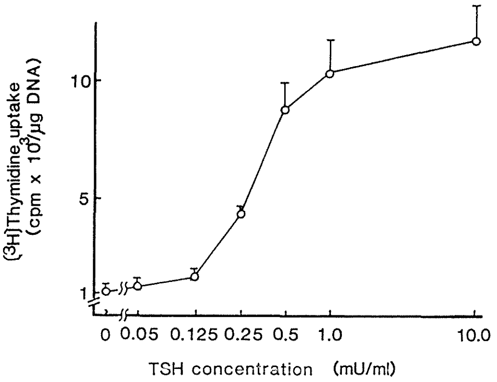

Fig. 2. Dose dependent stimulation of [^{3}H]thymidine incorporation into FRTL-5 cells by TSH. Cells were incubated for 24 h with varying concentrations of TSH. Each bar represents the mean ± SEM of values obtained in triplicate cultures.

Table 2. Effects of 8-bromo-cAMP, forskolin, and cholera toxin on [^{3}H] thymidine incorporation into FRTL-5 cells

Test material	[^{3}H] Thymidine (cpm/μg DNA)
Control	1146± 32.1*
8-brome-cAMP	
5 μg/ml	2481± 72.4**
15 μg/ml	3580±113**
30 μg/ml	6823±247**
50 μg/ml	9168±387**
Forskolin	
10^{-7} M	1627± 62.4**
10^{-6} M	10459±487**
5×10^{-6} M	12365±794**
10^{-5} M	12401±682**
Cholera toxin	
5×10^{-11} M	8684±139**
5×10^{-10} M	10028±250**
10^{-9} M	11483±250**
10^{-8} M	11728±195**

* Mean±SEM (3 determinations).

** $P<0.01$ vs. control.

Cells were grown in basal medium for 3 days. Each test material was present 1 μCi/ml [^{3}H] thymidine for the final 24 h.

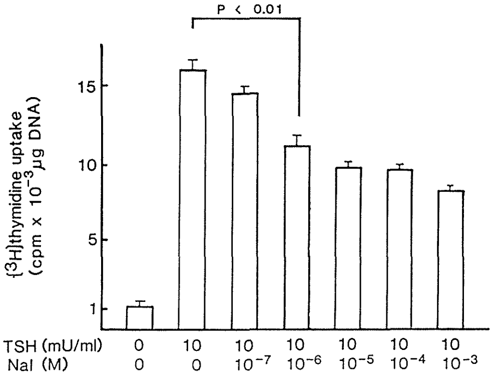

Fig. 3. Inhibitory effect of iodide on TSH-stimulated [^{3}H]thymidine incorporation into FRTL-5 cells. Cells were incubated with varying concentrations of iodide in the presence of TSH (10 mU/ml) for 24 h. Each bar represents the mean± SEM of values obtained in triplicate cultures.

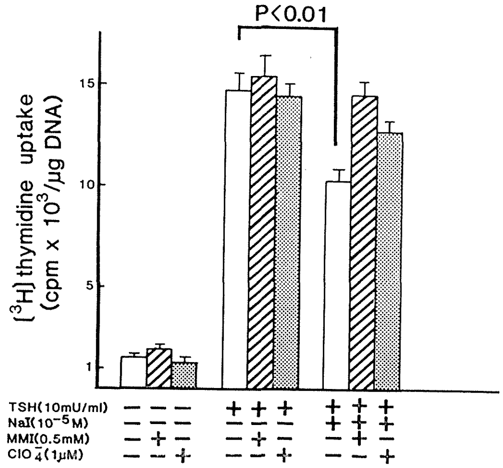

Fig. 4. Effect of methimazole (MMI) and ClO_4^- on iodide inhibition of [^{3}H]thymidine incorporation into FRTL-5 cells. MMI reversed, and ClO_4^- partially reversed the iodide effect. Both agents had no effect on the basal [^{3}H]thymidine incorporation. Each bar represents the mean±SEM of values obtained in triplicate cultures.

Table 3. Effect of iodide on TSH-stimulated cAMP release from FRTL-5 cells.

Additions	cAMP in the medium (pmol/ml)
Saline	4.0±0.5*
TSH	162 ±10
TSH+NaI (10^{-5}M)	87 ± 6**

* Mean±SEM (3 determinations).

** $P<0.01$ from the value of TSH.

Cyclic AMP was measured at 2 h as described in "Materials and Methods". To obtain the effect of iodide on the cAMP response to TSH, cells were preincubated for 12 h with or without NaI (10^{-5} M) in the presence of TSH (100 μU/ml). After removing the medium, the cells were further incubated for 2 h in 1 ml of HBSS containing 0.5 mM 3-isobutyl-1-methylxanthine and TSH (1 mU/ml).

Effects of iodide on [^{3}H]thymidine incorporation into DNA

FRTL-5 cells were exposed to varying concentrations of iodide, and [^{3}H]thymidine incorporation into DNA was determined. A significant inhibitory effect was seen

Table 4. Effect of iodide on the [^{3}H] thymidine incorporation stimulated by 8-bromo-cAMP, forskolin, and cholera toxin

Agents	[^{3}H] Thymidine incorporation (cpm/μg DNA)	
	Saline	10^{-5} M NaI
Control	1380± 29.5	1290± 31.3*
8-bromo-cAMP (50 μg/ml)	11247±593	9177±386***
Forskolin (5×10^{-6} M)	13524±621	8481±524**
Cholera toxin (5×10^{-10} M)	12351±718	8349±798***

* Means±SEM (3 determinations).

** $P<0.01$ vs. saline.

*** $P<0.05$ vs. saline.

Cells were grown in basal medium for 3 days. Each test material and iodide with 1 μCi/ml [^{3}H] thymidine incorporation were present for the final 24 h.

Table 5. Effect of iodide on FRTL-5 cell proliferation

Conditons	Cell number ($\times10^6$/well)
Basal medium	0.14±0.003*
+iodide (10^{-5} M)	0.13±0.004
TSH (10 mU/ml)	1.61±0.03**
+iodide (10^{-6} M)	1.44+0.03***
+iodide (5×10^{-6} M)	1.41±0.03***
+iodide (10^{-5} M)	1.38±0.04***
+iodide (10^{-5} M)+MMI (0.5 mM)	1.59±0.03

* Means±SEM (4 determinations).

*** $P<0.01$ vs. **.

Quadruplicate dishes of FRTL-5 cells and each additives, plated in 60 mm dishes at a cell concentration of $1\text{–}2\times10^5$/dish, were cultured for 5 days. They were then directly analyzed for growth by counting viable cells in a Neubauer chamber after release from plates by the 0.25% trypsin solution.

when the amount of iodide in the medium exceeded 10^{-6} M (Fig. 3). The fact that the inhibitory effect of iodide (10^{-5} M) was partially reversed by $NaClO_4$ (1 μM) suggests that iodide must enter the cells to act. Further, the iodide effect was abolished by the concurrent presence of 0.5 mM methimazole (MMI), an inhibitor of iodide organification (Fig. 4). In this experiment, increasing concentration of iodide had no significant effect on the basal [^{3}H]thymidine incorporation.

Role of cAMP in inhibitory effect of iodide

Iodide also inhibited TSH-stimulated cAMP release by FRTL-5 cells into the medium (Table 3).

To obtain further insight into the mechanisms by which iodide inhibited TSH-stimulated cAMP responses and thymidine uptake, 8-bromo-cAMP, forskolin and cholera toxin were used. They mimicked the action of TSH in stimulating [^{3}H]thymidine incorporation, and these stimulations were also suppressed by iodide (Table 4).

Effect of iodide on cell multiplication

The [^{3}H]thymidine incorporation into DNA does not necessarily reflect the number of cells undergoing a complete mitotic cycle, but is an indirect estimation of the number of cells that go through the 'S' phase of the mitotic cells. We tested, then, the inhibitory effect of iodide on TSH-stimulated cellular multiplication by directly counting cell numbers of cultures growing in the presence (10^{-6}–10^{-5} M) or absence of iodide (Table 5). Cell multiplication was significantly inhibited by iodide in the 5 day-culture. MMI also reversed the effect of iodide. Viability of the cells, assessed by nigrosine exclusion, was over 99%.

DISCUSSION

The function of thyroid cells is largely regulated by TSH through the receptor-mediated effect on adenylate cyclase. The role of cAMP as a positive signal for thyroid cell growth and proliferation was first reported by Roger *et al.*13) in dog thyroid cells in primary culture. Dere & Rapoport4) have suggested that cAMP is an essential intracellular mediator of TSH action in FRTL and FRTL-5 cells. A similar result, reported by Jin *et al.*10), indicated that $(Bu)_2$cAMP mimicked the TSH action in [^{3}H]thymidine incorporation into FRTL-5 cells.

All the key steps of iodide metabolism in the thyroid gland, (i.e. iodide transport, iodide binding to protein, hormone synthesis and secretion) are negatively regulated by high doses of iodide. However, the exact mechanism by which excess iodide inhibits a variety of thyroid functions is still not known. Many authors have shown that excess iodide affects the adenylate cyclase-cAMP system, and proposed this step as the main site of regulation. Van Sande and Dumont18,19) have reported that iodide inhibited the stimulatory effect of TSH on cAMP accumulation in canine thyroid slices in a completely *in vitro* system. We have also previously reported that thyroidal cAMP formation induced by thyrotropin *in vitro* was markedly inhibited by the acute administration of excess iodide to mice fed a low iodine diet9). Regarding the inhibitory action of iodide on thyroid growth, it is well known that treatment with Lugol solution decreases goiter size12). The regulation of some biochemical parameters related to thyroid growth, such as protein and RNA synthesis, has been reported11). Amino acid uptake by thyroid cells is inhibited by an organic iodine compound7). Valenta6) reported that iodine blocks protein biosynthesis in rat thyroid, while Pisarev & Aiello11) confirmed these results in calf thyroid. As shown in our results, iodide inhibited [^{3}H]thymidine incorporation into FRTL-5 cells in a dose dependent manner. This was confirmed in cell counting experiments. The concentration of extracellular iodide necessary for this effect is comparable to the concentration at which an effect is observed on other thyroid functions, including the iodide transport mechanism and adenylate cyclase activation by TSH. The iodide effect was partially reversed by $NaClO_4$ which suggests that iodide must enter the cell to act. A recent report by Becks *et al.*2) has presented almost identical data to ours, however, $NaClO_4$ did not reverse the inhibitory effect of iodide. They contribute this to an extracellular site of iodine organification14), although in our condition $NaClO_4$ was an effective inhibitor. MMI, an inhibitor of iodide organification, abolished the inhibitory effect of iodide, indicating that this phenomenon is dependent on the generation of an organic form of iodine. The sites of iodide inhibition of [^{3}H]thymidine incorporation was the first question to be asked in our investigation. It seems likely that the inhibition by iodide of cAMP production in our system is, at least in part, responsible for the iodide effect on [^{3}H]thymidine incorporation into FRTL-5 cells.

Further, [^{3}H]thymidine incorporation

stimulated by 8-bromo-cAMP was inhibited by iodide, indicates that iodide also acts on the steps after the cAMP formation. The data reported until now in different species, from *in vivo* and *in vitro* experiments, support the concept of an inhibitory effect of iodide at the level of cAMP formation, rather than on its degradation. The question of whether or not this iodide effect involves the specific TSH receptor was then studied. Forskolin and cholera toxin stimulation of $[^3H]$thymidine incorporation was tested for the inhibitory effect of iodide. It was found that stimulation by these agents was significantly depressed by excess iodide. Since the stimulation of $[^3H]$thymidine incorporation into DNA by these substances was also inhibited by iodide, the inhibitory action of iodide may be non-specific for TSH stimulation. Beyond this level, discrimination of the site of iodine action is difficult. The adenylate cyclase complex consists of a number of compounds, including the hormone receptor (R), intermediary regulatory proteins (Gs, Gi), and a catalytic unit (C). Filetti and Rapoport[6] reported that iodide exerts its inhibitory effect on adenylate cyclase at or near C. Our result that forskolin stimulation of $[^3H]$thymidine incorporation was inhibited by iodide indicates that C is one of the steps iodide acts on. The present study was not undertaken to carry out a detailed investigation of the mechanism by which organic iodine inhibits the $[^3H]$thymidine incorporation.

The second question raised was the amount of iodide organified in FRTL-5 cells. The present work supports the hypothesis that the inhibitory effect of iodide depends on its organification[7,9]. However, in FRTL-5 cells, only a small proportion taken up by the cells is organified[5]. Van Sande *et al.*[19] observed in dog thyroid slices, that the relation between the organified iodide and the magnitude of the inhibition on cAMP accumulation was not linear; the inhibitory effect plateaued earlier than the amount of iodide organified. Similarly, cooling greatly decreased total iodination while not relieving the inhibitory effect of iodide. They concluded that the iodination of the postulated pro-inhibitor is saturated earlier than general protein iodination. If iodine directly acts as an inhibitor, the mechanism of the effect of MMI must be clarified.

In summary, the iodide effect on adenylate cyclase activity in primary culture or slice systems can be duplicated in FRTL-5 cells. We also showed that iodide inhibited $[^3H]$thymidine incorporation into FRTL-5 cells, and cellular multiplication. The data demonstrate an autoregulatory role of iodide on the growth of FRTL-5 cells.

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