MODULATION OF ADENYLATE CYCLASE ACTIVITY BY THYROTROPIN IN CULTURED THYROID CELLS

Probable role of intracellular GTP

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1. Introduction

In cultured porcine thyroid cells the cyclic AMP response to a maximum concentration of thyrotropin (TSH), prostaglandin E_2 (PGE_2) or catecholamines is modulated both positively and negatively by the chronic presence of these stimulators in the culture medium [1–3]. When the chronic concentration of TSH was increased from 0–100 $\mu U/ml$, cells became increasingly sensitive to acute TSH or PGE_2 stimulation whereas their sensitivity to catecholamines was decreased. Higher TSH or PGE_2 concentrations induced homologous desensitization to the corresponding acute stimulator without further modification of the responsiveness to catecholamines.

To further characterize the mechanism of the metabolic effects induced by the chronic treatment of cells with TSH, it appeared important to study in cell-free system the two enzymes which regulate the intracellular cyclic AMP level, i.e., adenylate cyclase and phosphodiesterase.

We report here the results concerning adenylate cyclase. Hormonally-responsive adenylate cyclase is a multiple component system formed of at least 3 distinct units: a catalytic component containing the site for the cyclizing reaction; one (or several) hormone-specific receptor(s); and a regulatory component which binds guanyl nucleotide [4,5]. The positive effect induced by TSH on the cyclic AMP response over 0–100 $\mu U/ml$ [2] could be a consequence of modifications of one or several of these components. We show that:

(1) Stimulation of the adenylate cyclase of the

thyroid cell by the GTP analogue guanylylimido-diphosphate GppNHp is abolished in homogenates of cells chronically exposed to TSH;

(2) Such stimulation is restored when assayed in washed particulate preparations of the cyclase;

(3) The modulation of intracellular guanyl nucleotide content by chronic hormonal stimulation can account for these observations.

2. Materials and methods

2.1. Cell isolation and culture

Cells were isolated from thyroid glands of adult hogs by a modified [6] discontinuous trypsinization procedure [7]. The Eagle minimum essential medium used was buffered at pH 7.4 and supplemented with 10% newborn calf serum and contained 200 units/ml penicillin, 50 $\mu U/ml$ streptomycine sulfate and 0.25% (w/v) gelatin [8]. Freshly isolated cells (10 ml) were incubated at 2×10^6 cells/ml in polystyrene Petri dishes not treated for tissue culture to avoid cell attachment to the support and spreading. Incubation was done in the presence or absence of stimulator for 1–5 days at 37°C in 95% air/5% CO_2.

2.2. Fraction preparation

At the end of the incubation period the content of each dish was transferred into a centrifuge tube and diluted with 2 vol. Earle's salt solution buffered with 20 mM Hepes at pH 7.2 (Earle–Hepes). The suspension was spun at $250 \times g$ for 7 min at 4°C and the resulting pellet was suspended in Earle–Hepes and

washed once in the same buffer. The supernatant was discarded and the last drops were carefully wiped off with filter paper. The pellet was then suspended in 0.25 ml 10 mM Tris—Cl (pH 7.0) at 0°C and homogenized with 30 strokes of a hand-driven Teflon Potter homogenizer. The homogenate containing 4—6 mg protein/ml was immediately used or stored after freezing in liquid nitrogen (with no loss of activity). Subfractions of the homogenate were obtained by centrifugation at $10^4 \times g$ for 10 min. The $10^4 \times g$ pellet was washed once in the same conditions and the supernatant was spun at $10^5 \times g$ for 1 h to produce a $10^5 \times g$ supernatant.

2.3. Adenylate cyclase assay

Up to 0.10 mg protein of homogenate or $10^4 \times g$ pellet was incubated for 15 min at 30°C in 0.05 ml final vol. The medium contained 20 mM Tris—HCl (pH 7.8), 4.5 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM EGTA, 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), 20 mM creatine phosphate, 8 U/ml creatine kinase, 0.1% bovine serum albumin and 0.6—0.7 μCi [α-^{32}P]-ATP. Treatment of incubates and purification of the cyclic AMP produced was done using a slight modification [9] of the method in [10]. Values are given as means of duplicate determinations differing by ≤5%. Typical experiments on very similar 2—5 are described.

2.4. Other methods and chemicals

Particulate protein was solubilized in 2% SDS, 0.125 N NaOH and estimated by standard method [11]. Bovine TSH (1 U/mg) was from Armour (Chicago, IL), [α-^{32}P]ATP (30 Ci/mmol) from New England Nuclear (Dreieich) and trypsin, fetal calf serum and Eagle minimum essential medium from Grand Island Biological (Grand Island, NY). Biochem-

icals were from Boehringer (Mannheim) except IBMX which was from Aldrich (Milwaukee, WI). Other chemicals were of the highest purity commercially available.

3. Results and discussion

3.1. Basal and hormone stimulated adenyl cyclase activity of cell homogenates

Table 1 shows that basal adenylate cyclase activity of porcine thyroid cells was not changed upon culturing for 4 days in the absence (control cells) or presence of TSH at the two concentrations tested. Dramatic differences were observed for TSH- and isoproterenol-(IPNE) stimulated activities. After 4 days of culture in control conditions, the stimulability of adenylate cyclase by IPNE (ratio of hormone-stimulated to basal activity) was increased 5-fold whereas TSH stimulability remained constant. In contrast, cells cultured for 4 days in the presence of 100 μU/ml TSH exhibited a 5-fold higher adenylate cyclase stimulation by TSH than control cells at day 1 and a nearly constant stimulation by IPNE. These results agree with observations on modifications of cyclic AMP content in thyroid cells cultured in the presence of TSH or IPNE [1—3].

3.2. Effects of guanylylimidodiphosphate on the basal and hormone-stimulated adenylate cyclase activities of cell homogenates

The effect of GppNHp on basal and hormone-stimulated adenylate cyclase activities is illustrated in table 1. GppNHp alone or in association with TSH is an effective activator of adenylate cyclase from control cells cultured for 1 or 4 days. In contrast no effect was observed in cells cultured for 4 days in the presence of TSH (0.1 or 1 mU/ml).

Table 1
Adenylate cyclase activity of homogenates of thyroid cells cultured for 1 and 4 days in the presence or absence of thyrotropin

TSH in culture medium, no. days in culture	Adenylate cyclase activity in 15 min (pmol cyclic AMP . min^{-1} . mg protein^{-1})						
	Basal	GppNHp-stim.	TSH-stim.	TSH- + GppNHp-stim.	IPNE-stim.	IPNE - + GppNHp-stim.	NaF-stim.
None, 1 day	2.2	13.2	5.2	17.6	5.7	17.7	27.5
None, 4 days	2.8	14.2	5.0	29.0	24.0	55.0	43.6
0.1 mU TSH/ml, 4 days	2.3	2.5	24.2	19.6	5.5	7.7	32.1
1 mU TSH/ml, 4 days	2.4	3.2	5.5	10.2	4.6	6.0	26.2

Concentration of stimulators added in the adenylate cyclase assay: GppNHp, 10 μM; TSH, 25 mU/ml; IPNE, 10 μM; NaF, 10 mM

3.3. Chronic effects of graded concentration of thyrotropin

In order to further characterize the TSH effect, adenylate cyclase stimulation of thyroid cells was tested after 4 days of culture in the presence of increasing concentrations of TSH. As shown in fig.1A, the acute stimulation of adenylate cyclase by TSH was maximum when cells were cultured in the presence of 0.1 mU/ml TSH. Very low stimulation was observed in cells cultured with ⩾0.5 mU TSH/ml. In contrast (fig.1B), the sensitivity to acute isoproterenol stimulation was maximum in control cells and disappeared completely upon chronic treatment with 0.1 mU TSH/ml and higher. Adenylate cyclase stimulation by GppNHp calculated as the ratio of GppNHp-stimulated level to basal level or as the ratio of GppNHp + hormone-stimulated level to hormone-stimulated level is shown in fig.1C. A maximum effect of GppNHp was always observed with homogenates derived from cells cultured in control medium. The GppNHp effect decreased when chronic TSH concentration in the culture medium increased up to 0.1 mU/ml.

3.4. Kinetics of acute stimulatory effects

Fig.2 shows the linear time-course of adenylate cyclase activity of homogenates of cells cultured for 4 days in the presence of 0.1 mU/ml TSH. No detectable effect of GppNHp on the basal or TSH-stimulated level occurred during the first 12 min incubation.

Fig.2. Time-course of cyclic AMP production by homogate of thyroid cells cultured for 4 days in the presence of 0.1 mU TSH/ml. Homogenates were incubated without effector (●) or with 10 μM GppNHp (○), 25 mU/ml TSH (△) or 25 mU/ml TSH + 10 μM GppNHp (▲).
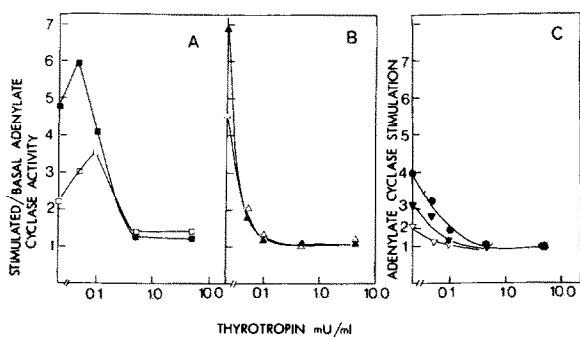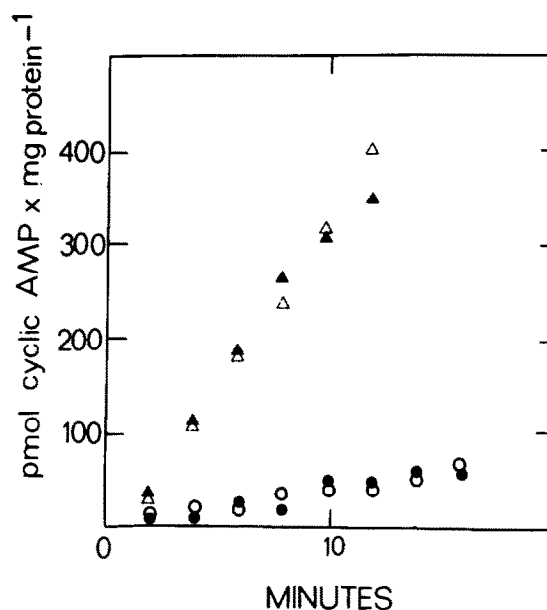

Fig.1. Adenylate cyclase stimulation of homogenates of thyroid cells cultured for 4 days with increasing concentrations of thyrotropin. Stimulation of the homogenate with: (A) 25 mU/ml TSH (□), 25 mU/ml TSH + 10 μM GppNHp (■); (B) 10 μM IPNE (△), 10 μM IPNE + 10 μM GppNHp (▲); (C) GppNHp-stimulated level: basal level (●), GppNHp + TSH-stimulated level:TSH level (▼); GppNHp + IPNE-stimulated level:IPNE-stimulated level (▽).

3.5. Basal and stimulated adenylate cyclase activity of 10^4 × g-particulate fractions

The absence of response of the enzyme to GppNHp in homogenates of cells chronically cultured in the presence of TSH might result from structural modifications of adenylate cyclase or from changes in the coupling state of the enzyme related to interaction with soluble intracellular factor(s) [12–14]. To test this possibility, the adenylate cyclase activity of a 10^4 × g-particulate fraction containing all the enzyme activity of the homogenate was derived from the homogenate of cells cultured in the presence of 0.1 mU/ml TSH and compared to the activity of the total homogenate. Table 2 shows that the stimulation of adenylate cyclase by GppNHp or GppNHp + TSH was restored when the enzyme activity was tested in the 10^4 × g particulate fraction and that stimulation by TSH alone was greatly lowered. Addition to the 10^4 × g particulate fraction of the 10^5 × g supernatant derived from the same homogenate gave a pattern of stimulation nearly identical to that of the whole homogenate. Moreover, the addition of 10 μM GTP to the adenylate cyclase assay of the 10^4 × g fraction abolished the GppNHp effect and restored

Table 2

Adenylate cyclase stimulation of the homogenate and a $10^4 \times g$ particulate fraction from thyroid cells cultured for 4 days in the presence of 0.1 mU TSH/ml

Fraction	Stimulator		
	GppNHp (10 μM)	TSH (25 mU/ml)	GppNHp (10 μM) + TSH (25 mU/ml)
Homogenate	1.2	5.6	6.1
$10^4 \times g$ pellet[a]	6.2	2.2	15.7
$10^4 \times g$ pellet + $10^5 \times g$ supernatant	1.0	4.0	4.0
$10^4 \times g$ pellet + 10 μM GTP	1.4	5.3	

[a] Amount of $10^4 \times g$ pellet and/or $10^4 \times g$ supernatant corresponding to that contained in the aliquot of homogenate tested

Stimulation is defined as the ratio of stimulated to basal adenylate cyclase activity. The $10^4 \times g$ pellet contained 50% of the total protein content of the homogenate and all its adenylate cyclase activity

its stimulation by TSH alone. At 10 μM GTP was ineffective in inducing significant modification of basal adenylate cyclase activity (not illustrated). Preliminary experiments showed that the factor present in the supernatant was heat stable and dialyzable.

Thus the increased responsiveness of the adenylate cyclase system to TSH (positive regulation) observed when thyroid cells are cultured with 0–100 μU/ml TSH ([2,3], table 1) might be associated with modulation of the intracellular GTP level by the hormone. An estimate of ~1 mM for the intracellular GTP concentration in TSH-treated cells can be derived from data of the type described in table 2 if we assume that the concentration of free GTP in the homogenate is 10 μM on the basis of the identical effect obtained on TSH stimulation of the $10^4 \times g$ fraction by addition of the $10^5 \times g$ supernatant or 10 μM GTP and using 4 μl/mg protein for the cell volume [15]. A 5–10-fold lower value for GTP concentration can be approximated for control thyroid cells taking into account the dose–effect curve of the inhibitory action of GTP on the stimulation of adenylate cyclase by GppNHp (unpublished). The role of GTP in the regulation of adenylate cyclase in vitro is now well documented [4,5]. Hormonal activation of adenylate cyclase from different tissues was dependent upon cytosolic factors [12–13] and the epinephrine stimulation of membrane adenylate cyclase by cytosol could be related to the presence of guanyl nucleotide in the soluble fraction in a human astrocytoma cell line [14]. The participation of GTP in important cel-

lular functions such as protein synthesis and microtubule assembly suggests that the control of intracellular GTP level by TSH could play a central role in the chronic action of the hormone.

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