NOTE

Release of Cyclic AMP from the Chicken Thyroid Stimulated with TSH *In Vitro*

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Synopsis

The thyroid lobes excised from one-day-old or 13-day-old chickens were incubated with or without TSH, and cAMP in the medium was determined. TSH induced the release of cAMP from thyroid lobes, resulting in the increase of cAMP concentration in the incubating medium. The release of cAMP into the incubating media in the presence of TSH from the thyroids of the chickens which were pretreated with TSH neonatally was more marked as compared to that from the thyroids of the control chickens. The light and electron microscopic examination of the thyroid lobes incubated with TSH showed that the morphological changes such as colloid droplets formation and enlargement of endoplasmic reticulum were induced.

It is now generally accepted that the primary effect of TSH on the thyroid is to activate adenylate cyclase and to raise the cyclic 3', 5'-adenosine monophosphate (cAMP) in the follicular cells. This is in accordance with the second messenger scheme of cAMP function in other hormonal actions which are mediated by adenylate cyclase system. Recently, however, reports appeared showing the release of cAMP into the perfusion medium; in the liver stimulated by glucagon (Kuster *et al.*, 1973), in the adipose tissue stimulated by epinephrine (Zumstein *et al.*, 1974) and in the ovary stimulated by human chorionic gonadotrophin or prostaglandin (Liljekvist *et al.*, 1976). It should be remembered that many early experiments which were carried out in order to prove the mediation of cAMP in hormonal effects involved the demonstration of the mimic responses elicited by the extracellular addition of

cAMP or its dibutyryl derivative. The possibility may exist that the extracellular cAMP extruded from cells has some physiological significance if the cAMP release is observable in many hormonal effects which are mediated by adenylate cyclase system and proved not to be an artifact due to the lesion of cells during incubation.

The present study demonstrates that cAMP is released into the medium from chicken thyroid gland incubated *in vitro* with TSH. Chicken thyroid is suitable for this experiment because the lobes located separately and are excised easily as being tissue trauma avoided.

Materials and Methods

Thyroid glands from male White Leghorn chickens which were one day old or 13 days old were used. The chickens were reared in a brooder with food and water supplied *ad libitum*. After decapitation, thyroid lobes were excised with minimal direct manipulation in order to avoid tissue trauma. In-

cubation procedure was essentially the same as that described previously (Tonoue *et al.*, 1970). It has been confirmed that the thyroid gland responds to added TSH in the hormone release and cAMP formation activity in this incubating system (Tonoue *et al.*, 1970). Excised lobes were pooled in the weighed test tubes (12×75 mm) so that the weight of lobes exceeded 25 mg a little. The test tubes contained 0.75 ml of ice-cold Medium 199 (Hanks BSS, Chiba-Ken Kessei Kenkyusho), pH 7.4, buffered with sodium bicarbonate saturated with 95% O₂ and 5% CO₂, and supplemented with theophylline at the concentration of 0.7 mM. The weights of single lobes of one-day-old and 13-day-old chicken were 1.5–2.0 and 3.5–4.1 mg, respectively. The incubation was carried out at 37°C under a gas phase of 95% O₂ and 5% CO₂ with shaking at 100 oscillations per min. After 10 min-temperature equilibration, 50 μ l of medium was taken out to serve as 0 time sample for cAMP assay. Immediately after 0 time sample was taken out, 50 μ l of fresh medium with or without 150 mU of bovine TSH (Thytropar, Armour) or 100 μ g of ACTH (Acthar, Armour) was added. Thereafter, 50 μ l samples of incubating medium were taken at time intervals as shown in the Figures. In the experiment demonstrating the cAMP release from a single lobe, each lobe was incubated for 30 min in the small test tube (7×50 mm) containing 50 μ l incubating medium with or without TSH and cAMP in the medium was assayed after the lobe was taken out. The collected samples in the small test tubes were frozen by dry ice as quick as possible and stored at –25°C until assayed. Cyclic AMP was determined by the competitive protein binding assay method using a cAMP assay kit purchased from the Radiochemical Centre, Amersham, England (Code, TRK 432).

In an attempt to induce the change in responsiveness of the thyroids to TSH, some birds were pretreated with TSH neonatally. They were intraperitoneally injected with 40 mU of bovine TSH (Thytropar, Armour) in 0.1 ml saline on the first and third day post hatch and were sacrificed on the 13th day. The thyroid lobes were fixed by the Bouin solution at the termination of incubation. Paraffin-embedded blocks were cut in serial 8- μ m sections and they were stained with periodic acid Schiff. For electron microscopic observation, the thyroids of one-day-old chickens were incubated in the same way as described before and the lobes were picked up at 10, 30 and 45 min of incubation and transferred into 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.3 and dissected into pieces there. After dehydration in ethanol the specimens were embedded in Epoxy resin. Ultrathin sections were cut on a Porter-Blum MT-1 ultramicrotome and mounted on formvar-coated copper grids. The sections were stained with uranyl acetate and lead citrate. The specimens were examined in a HITACHI 11DS electron microscope.

Results

Cyclic AMP concentration in the incubating medium did not increase during incubation without TSH. TSH added at the concentration of 200 mU/ml induced the release of cAMP into the medium from the thyroid lobes while ACTH did not elicit any release of cAMP (Figs. 1 and 2). The increase in cAMP concentration in the medium was almost linear for the first 45 min and seemed to level off thereafter. The response to TSH in cAMP release was more remarkable in the thyroid of TSH-pretreated 13-day-old chicken than that of normal 13-day-old chicken (Figs. 1 and 2). This was confirmed statistically in

Fig. 1. Change in cAMP concentration in the medium during incubation of thyroid lobes from 13-day-old chickens. Incubation of 27 mg thyroid lobes.

Fig. 2. Change in cAMP concentration in the medium during incubation of thyroid lobes from 13-day-old chickens pretreated with TSH neonatally. Incubation of 28 mg thyroid lobes.

Table 1. *In vitro* effect of TSH on the cAMP release from chicken thyroid lobes.

Pretreatment	Addition	No.	cAMP in the medium after 30 min incubation n moles/g lobes, mean $\pm$ SE.	P
None	None	6	0.23 ± 0.03	
None	TSH 200 mU/ml	6	0.86 ± 0.09	0.01
TSH	None	6	0.33 ± 0.04	0.01
TSH	TSH 200 mU/ml	6	1.70 ± 0.35	0.01

Thyroid lobes from 13-day-old chicken were incubated individually in 50 μ l medium. TSH pretreatment; injection of 40 mU TSH on the first and third day post hatch. Thyroid lobe weight; no pretreated 3.7 ± 0.1 mg, TSH pretreated 3.8 ± 0.2 mg.

Fig. 3. Change in cAMP concentration in the medium during incubation of thyroid lobes from one-day-old chickens. Incubation of 27 mg thyroid lobes.

the experiment of incubating a single lobe separately for 30 min with or without TSH (Table 1).

The increase in the cAMP concentration in the medium of incubation with TSH of thyroid from one-day-old chicken was also remarkable (Fig. 3).

The light and electron microscopic examination of incubated thyroid tissues affirmed no lesions in the thyroid tissue. An increase in the number of cells containing colloid droplets was observed in all the lobes incubated with TSH. The most remarkable response to TSH in the colloid droplet formation was observed in the thyroid of TSH-pretreated, 13-day-old chicken (Fig. 4).

Fig. 4. Intracellular colloid droplets in the thyroid gland incubated for 60 min with or without TSH. 1a, without TSH. 1b, with TSH. $\times 430$. The thyroid glands were excised from the 13-day-old chicken pretreated with TSH neonatally.

The electron microscopic examination (Fig. 5) showed that the fine structures of the one-day-old chicken thyroids incubated for 45 min without TSH were within the normal range compared to those of the non-incubated thyroids; the colloid droplets were scarcely appeared, but more or less

dense granules were detected to be scattered over the apical region of the cells. In the thyroids incubated with TSH for 45 min, many colloid droplets appeared in the apical or lateral cytoplasm, and the cisternae of rough surfaced endoplasmic reticulum was prominently dilated. The findings showed that Golgi apparatus, microvilli and pseudopod were neither enough developed, nor was shown the close local affinity of colloid droplets to dense bodies.

Discussion

The present results demonstrated that cAMP was released from thyroid lobes which were incubated with TSH *in vitro*, similarly as from the liver cells, adipose tissue and ovary which were superfused with the respective stimulating hormones (Kuster *et al.*, 1973); (Zumstein *et al.*, 1974); (Liljekvist *et al.*, 1976). The possibility that the cAMP was extruded from the adherent fat tissue was ruled out since ACTH was not effective.

Many studies have been made on *in vivo* effect of TSH on thyroid ultrastructure but studies *in vitro* were scarce. Neve and Dumont (1970) reported the ultrastructural changes in dog thyroid slices incubated with TSH, cAMP, dibutyl cAMP or prostaglandin E₁ for 60 min. Those changes were pseudopod formation and colloid droplet engulfment with no migration of dense bodies or dilation of the rough surfaced endoplasmic reticulum. In the present experiment, colloid droplet formation and dilation of the cisternae of rough surfaced endoplasmic reticulum were remarkable but pseudopod formation was not so prominent. Those changes correspond, partly at least, with the features observed previously by Fujita (1963) in the thyroid of TSH-injected chicken and seems to show that the reabsorption of colloid and the formation of thyroglobulin or other proteins was stimulated by TSH (Fujita, 1975). From these histological examinations it may be concluded that the cAMP release from the thyroid is the normal response of the follicular cells and not resulted from any artifactual morphological changes which might be supposed to occur during the incubation with TSH *in vitro*. The increase of colloid droplets without prominent pseudopod formation seems to suggest that the process other than the engulfment by pseudopod of colloid reabsorption, as speculated

Fig. 5. A part of ultrastructure of the follicular cells from the one-day-old chicken thyroids incubated for 45 min with or without TSH. 2a, without TSH. 2b, with TSH. $\times 11000$. ger, rough surfaced endoplasmic reticulum. G, Golgi area. d, colloid droplet. n, nucleus. m, mitochondria.

by Seljelid *et al.* (1970), was stimulated by TSH in the present experiment. The fact that the thyroid from TSH-pretreated chicken was more responsive to TSH in both cAMP release and colloid droplet formation than that from control chicken seems to indicate the possible relationship between the cAMP release and the activity of the follicular cells.

It is an interesting result that the thyroid from one-day-old chicken showed more marked cAMP release response to TSH than that of 13-day-old chicken. This might be due to the difference in the size of thyroid lobe, but the result is compatible with the recent finding by Davison (1976). He reported that plasma thyroxine level showed a peak in the one-day-old chicken and decline in the week after hatching. He also described that the maximum response to exogenous TSH was observed within a few hours in the neonate chicken.

The interest on the functional significance of the presence of the extruded extracellular cyclic nucleotides was expressed already by Hardman in the book by Sutherland and his colleagues (1971). However, little studies on the extracellular cAMP were available with respect to its physiological significance in the TSH effect on the thyroid. If we suppose that the figures in Table 1 reflect the release rate of cAMP, they were 10 to 25 % of the accumulation rate of intracellular cAMP in the mammalian thyroid which was stimulated by TSH *in vitro* (Tonoue *et al.*, 1970; Ketelbant-Balasse *et al.*, 1976).

In aggregating cells of the slime mold, it has been proposed that each cell acts as a biochemical signal amplifier so that extracellular cAMP would play the role of transmitter in a relay system (Roos *et al.* 1975). Though the present results of cAMP release do not allow to indicate any role of

extracellular cAMP in the thyroidal response to TSH, it should be taken into account in the study of the mode of action of TSH on the thyroid. Recently, Verrier and Cailla (1976) investigated the response of cultured thyroid cells to TSH and found that only cells cultured in the presence of dibutyryl cAMP retain the capability of increasing their cAMP concentration in the cells following TSH stimulation whereas the cells cultured in the absence of dibutyryl cAMP do not preserve this quality.

At least, cAMP release may be related to the intracellular cAMP concentration. It was reported that the thyroidal intracellular cAMP was not always increased by chronic propylthiouracil treatment in the rat in spite of drastic increase in circulating TSH level (Nakamura *et al.*, 1976).

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