Effect of Thyroliberin on the Concentration of Adenosine 3':5'-Phosphate and on the Activity of Adenosine 3':5'-Phosphate-Dependent Protein Kinase in Prolactin-Producing Cells in Culture

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1. The effects of thyroliberin were studied in cultured rat pituitary-tumour cells that synthesize and secrete prolactin (the GH₄C₁ cell strain). 2. Prolactin and cyclic AMP were measured by radioimmunological methods, and a cyclic AMP-dependent protein kinase was characterized by using histone as substrate. 3. Prolactin release was studied after 5–60 min of treatment, and synthesis after 48 h of treatment with thyroliberin. One-half maximum stimulation of release and synthesis were observed at 0.25 and at 4 nM respectively. 4. Cyclic AMP was temporarily increased in cell suspensions after treatment with thyroliberin, and one-half maximum stimulation was observed at 25 nM. 5. Dibutyl cyclic AMP increased prolactin release and synthesis, one-half maximum effects being obtained at 20 μ M. 6. A cyclic AMP-dependent protein kinase, which was one-half maximally stimulated at 30 nM-cyclic AMP, was demonstrated. 7. An increase in the activity ratio (–cyclic AMP/+cyclic AMP) of the cyclic AMP-dependent protein kinase was observed after treatment with thyroliberin. Total protein kinase activity in the presence of cyclic AMP was unaltered. The time-course of enzyme activation was similar to that of cyclic AMP formation and corresponded to the time when prolactin release was first observed. 8. It is concluded that thyroliberin induces cyclic AMP formation, resulting in the activation of a cyclic AMP-dependent protein kinase.

Several reports (Bowers, 1971; Tal *et al.*, 1974; Labrie *et al.*, 1975) show that the hypothalamic tripeptide thyroliberin increases concentrations of cyclic AMP or incorporation of [¹⁴C]adenine into cyclic AMP in explants of adenohypophysis. The tissue used contains at least two functionally different cell types that are sensitive to thyroliberin, since the tripeptide causes release of both thyrotropin and prolactin (Bowers *et al.*, 1971; Frantz *et al.*, 1972; Schally *et al.*, 1973; Vale *et al.*, 1973; Porteus & Malven, 1974; Smith & Convey, 1975). Also, cyclic nucleotide analogues stimulate the release of both hormones (Cehovic, 1969; Wilber *et al.*, 1969; Bowers, 1971; Wakabayashi *et al.*, 1973; Labrie *et al.*, 1973; Schally *et al.*, 1973). Therefore cyclic AMP has been suggested to mediate the actions of thyroliberin (Robison *et al.*, 1971; Hardman, 1974). However, the heterogeneity of the tissue used complicates an analysis of a possible causal relationship between cyclic AMP formation and the biological effect of thyroliberin on thyrotropin and/or prolactin.

In the present study we have used a homogeneous system of cultured rat pituitary cells (GH₄C₁ cells) that only synthesize and secrete prolactin (Tashjian & Hoyt, 1972; Haug & Gautvik, 1976). We have

measured separately the effects of thyroliberin on prolactin release and synthesis and on cellular concentrations of cyclic AMP. In the same cell suspensions we have characterized a cyclic AMP-dependent protein kinase, which is activated by pretreatment of the cells with thyroliberin.

Materials and Methods

Culture of the GH₄C₁ cells

The GH cells are originally derived from a rat pituitary tumour, and the establishment and culture of these cells have been described previously (Tashjian *et al.*, 1968). The GH₄C₁ cells represent a sub-clone that only synthesizes and secretes prolactin (Tashjian & Hoyt, 1972; Haug & Gautvik, 1976). About two-thirds of cells in exponentially growing monolayer cultures synthesize and secrete the hormone (Gautvik & Fossum, 1976). In the present study the cells were cultured as monolayers in plastic tissue-culture dishes (Falcon, Oxnard, CA 93030, U.S.A.) containing Ham's F-10 medium (Ham, 1963) supplemented with 15 and 3% (w/w) foetal calf serum (Flow Laboratories, Irvine, Scotland, U.K.). The cells were incubated at 37°C in a humidified

atmosphere of $O_2 + CO_2$ (95:5) and the experiments were performed with cells in the exponential phase of growth. Medium was always changed 24h before the experiments were started.

Cell suspensions were used for measurements of hormone release, and monolayer cultures were used for studying prolactin synthesis.

Measurements of prolactin release and synthesis

Newly synthesized prolactin is released into the medium 30min after addition of labelled amino acids to monolayer cultures of GH_4C_1 cells (Gautvik *et al.*, 1976). Thyroliberin increased synthesis of prolactin after 4–5h of treatment (Dannies & Tashjian, 1973; Gautvik & Kriz, 1976b). Therefore prolactin release was usually measured in the medium after 20–30min incubation. Under these conditions the release of already synthesized and stored hormone was studied. Samples of 100 μ l of cell suspensions (150–300 μ g of cell protein) in serum-free medium (Nueman-Tytel; Grand Island Biological Co., Grand Island, NY, U.S.A.) were added to buffer [300 μ l of 154mM-NaCl/10mM-Tris/HCl/1.0mM-EDTA (sodium salt) (pH7.5)]. Before the addition of cells 1.5mM-MgCl₂ was added to each tube. Thyroliberin (a gift from F. Hoffmann-LaRoche and Co. A.G., Basel, Switzerland) and dibutyl cyclic AMP (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used in the concentrations indicated. The incubations were performed at 37°C in a shaking water bath for the times indicated. The experiments were stopped by placing the tubes on ice and centrifugation at 500g for 1 min at 0°C.

The total amount of hormone that accumulated in the medium during 24h of incubation was used as a measure of prolactin synthesis for the following reasons. (1) The intracellular stores of prolactin are small (Gautvik & Kriz, 1976a; Haug & Gautvik, 1976). (2) Since the rate of prolactin synthesis is high (Gautvik & Kriz, 1976a) most of the newly formed hormone is released, and after 24h of incubation a very small fraction of the total hormone in the system remains intracellularly. (3) Prolactin is not degraded intracellularly (Dannies & Tashjian, 1973) and the hormone is stable in the culture medium during the experimental period (Tashjian *et al.*, 1970).

Before an experiment was started the cells were washed twice with medium pre-equilibrated in $O_2 + CO_2$ at 37°C for 2h, followed by the addition of 2ml of culture medium.

Radioimmunoassay of prolactin

Total intra- and extra-cellular amounts of prolactin were measured with a specific radioimmunoassay by using the double-antibody technique (Haug & Gautvik, 1976). The smallest amount of prolactin

that could be detected was 3.0ng/ml. When necessary the culture medium was diluted with assay buffer (0.05M-NaH₂PO₄/0.1M-NaCl/0.05% NaN₃) containing 1% (v/v) horse serum. Each determination was performed in duplicate.

Measurements of cyclic AMP

The incubation mixture was the same as when prolactin release was studied, and the total volume of each tube was 400 μ l. The reaction was initiated by the addition of cells to prewarmed tubes (37°C) standing in a shaking water bath. After different incubation periods the reaction was stopped by the addition of 100 μ l of 30% (w/v) trichloroacetic acid followed by 100 μ l of 1M-HCl, and 10000c.p.m. of cyclic [³H]AMP for calculation of recovery. The tubes were centrifuged and the supernatants extracted with water-saturated diethyl ether as described previously (Gautvik & Kriz, 1976c). The pellets were used for protein measurements. The Schwarz/Mann (Orangeburg, NY, U.S.A.) kit was used as indicated by the makers for measurements of cyclic AMP, and the duplicate determinations differed by less than 15%.

Determination of cyclic AMP-dependent protein kinase

Cell suspensions containing 2–3mg of protein were centrifuged at 3000g and 4°C for 10min and the supernatants discarded. The pellets were homogenized at 0°C in 3ml of 50mM-Tris/HCl buffer, pH7.4, containing 4mM-EDTA by using a glass homogenizer (20 strokes with a motor-driven Teflon pestle). Samples of the homogenates were added to the incubation mixture. Control experiments showed that cellular cyclic AMP was diluted to such an extent (<10nM) during the homogenization procedure that dissociation of protein kinase due to endogenous cyclic AMP was negligible.

Protein kinase was assayed by two different procedures.

Method 1. The enzyme activity was assayed by the method of Reimann *et al.* (1971) with the modifications described previously (Walaas *et al.*, 1973). The assay system contained, in a total volume of 200 μ l: 100 μ g of histone (type A11; Sigma), 10mM-MgCl₂, 0.2mM-[γ -³²P]ATP (specific radioactivity 16Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.), 8mM-theophylline, 1mM-EDTA, 2mM-dithiothreitol, 20mM-NaF, 1 μ M-cyclic AMP and 50 μ l of cell homogenate (containing usually 50 μ g of protein). The incubations were usually performed at 30°C for 10min. The reactions were terminated by spotting samples of the reaction mixture on to filter paper, and transferring the filter papers after washing to liquid-scintillation vials containing 10ml

of Instagel (Packard Instrument Co., La Grange, IL, U.S.A.).

Method II. The assay system contained, in a total volume of 100 μ l: 50 μ g of histone and additions as given above for method I. The reaction was initiated by addition of 15 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Incubations were performed at 30°C for 60s, and the reaction was terminated by addition of 50 μ l of stop solution containing 9% (w/v) sodium dodecyl sulphate (Sigma), 3mM-Tris/HCl, pH7.8, and 3mM-EDTA. The mixture was heated in a boiling-water bath for 2min to eliminate proteolytic activity and incubated with 150mM-dithiothreitol for 60min at 37°C. Samples (20 μ l) containing 7 μ g of histone were then subjected to electrophoresis on sodium dodecyl sulphate/polyacrylamide-slab gels by the method of Fairbanks *et al.* (1971).

The gels were stained with Coomassie Blue (RDS-L, Canaco Inc., Rockville, MD, U.S.A.) and destained. The arginine-rich histone bands (mol.wt. 17500) were removed from the gels and sliced into 2mm slices. The gel slices were decolorized and dissolved by adding 1ml of Soluene 350 and 0.5ml of H_2O_2 (30%, v/v) and kept at 60°C for 3h. Scintillation fluid (10ml) was added to the vials that were kept overnight before counting for radioactivity in a Packard spectrometer.

The $[\text{P}^{32}]$ phosphorylated histone has been separated by method II from other phosphorylated proteins (endogenous substrates) before counting the radioactivity, resulting in lower 'background' counts. Method II is more time-consuming than method I, but has the advantage of greater sensitivity, so that shorter incubation time (60s) could be used.

Cell protein

This was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V, powder; Sigma) as standard.

Statistical analysis

The two-sided non-parametric rank test of Wilcoxon was used for statistical analysis. In addition, the mean values and standard errors of the data are indicated on the Figures.

Results

Effect of thyroliberin on prolactin release and synthesis

Fig. 1 shows that thyroliberin (3 μ M) stimulates the release of prolactin from suspension of GH_4C_1 cells as it does in monolayer cultures of a related cell strain producing both prolactin and growth hormone (Dannies *et al.*, 1976). The first significant effect on prolactin release was observed after 5 min ($P < 0.05$),

Fig. 1. Time-course of effects of thyroliberin on prolactin release

Suspension of cells (100 μ l) were added to tubes containing buffer without (○) and with thyroliberin (●) (3 μ M), and incubated at 37°C for the times indicated (see the Materials and Methods section). After the experiment the tubes were removed and placed on ice, centrifuged and prolactin was measured in the supernatant. The results are expressed as means $\pm$ S.E.M. for four tubes.

Fig. 2. Dose-response curve for the effect of thyroliberin on prolactin release

Suspensions of cells (100 μ l) were added to tubes containing buffer (see the Materials and Methods section) without or with the indicated concentrations of thyroliberin and incubated at 37°C for 30min. Prolactin was measured in the incubation medium after centrifugation as described in legend to Fig. 1. The results are expressed as means $\pm$ S.E.M. for three tubes.

and the difference between treated and control group increased up to 60min. From 0.5 to 2min of incubation there were no changes in the release of prolactin, and the hormone values were identical in the two

Fig. 3. Dose-response curve for the effect of thyroliberin on prolactin synthesis

Monolayer cultures received thyroliberin for 48 h at the concentrations indicated. Prolactin was measured in the medium by using radioimmunoassay as described in the Materials and Methods section. The results are expressed as means $\pm$ S.E.M. for three dishes, and hormone values for the last 24 h of incubation are shown.

Fig. 4. Time-course of the effect of thyroliberin on cyclic AMP formation

Cell suspensions (160 µg of cell protein/100 µl) were added to tubes prewarmed at 37°C with ($\circ$) and without (Δ) thyroliberin (3 µM). Control and treated tubes were harvested at the times indicated, and cyclic AMP was measured as described in the Materials and Methods section. The results are given as means $\pm$ S.E.M. for three tubes.

groups. Control experiments have shown that this value represents the amount of prolactin that accumulated in 100 µl of the stock-cell suspension during the time that elapsed before the start of the experiment. Since no corrections have been made for

Fig. 5. Dose-response curve for the effects of thyroliberin on cyclic AMP formation

Cell suspensions (190 µg of cell protein/100 µl) were added to prewarmed tubes at 37°C without or with the concentrations of thyroliberin indicated. The tubes were harvested after 5 min of incubation, and cyclic AMP was measured as described in the Materials and Methods section. The results are given as means $\pm$ S.E.M. for four tubes.

this background value, the relative differences between the control and the treated groups in the release experiments are underestimated. Measurements of intracellular prolactin during three experiments showed a corresponding decrease, so that total amount of hormone in the system remained constant (results not shown).

Fig. 2 shows that the dose-response curve of thyroliberin on prolactin release in suspensions of GH₄C₁ cells reaches maximum values at about 1 nM and one-half maximal value at about 0.25 nM. The concentration required for one-half maximum effect of thyroliberin on prolactin release is different from that (4 nM) required for the equivalent effect on prolactin synthesis (Fig. 3).

Effects of thyroliberin on the concentration of cyclic AMP

Fig. 4 shows that thyroliberin (3 µM) caused a transient increase in cyclic AMP concentrations measured as total intra- and extra-cellular cyclic nucleotide (see the Materials and Methods section). Significant increase in cyclic AMP was observed 30 s after the start of incubation, and maximal values were

Fig. 6. Effect of dibutyryl cyclic AMP on prolactin release

Suspensions of cells (100 µl) were added to tubes containing the indicated concentration of dibutyryl cyclic AMP. Prolactin was measured in the supernatant after incubation at 37°C for 20 min and centrifugation as described in the Materials and Methods section. The results are given as means $\pm$ S.E.M. for three or four tubes.

Fig. 7. Effect of dibutyryl cyclic AMP on prolactin synthesis

Monolayer cultures received dibutyryl cyclic AMP at the concentrations indicated for 53 h. Extracellular hormone accumulation was measured during the last 5 h of incubation as described in the Materials and Methods section. The results are presented as means $\pm$ S.E.M. for four to six dishes.

obtained after 5 min. The dose-response curve showed that about 25 nM-thyroliberin gave one-half maximal increases in cyclic AMP formation (Fig. 5).

Effects of dibutyryl cyclic AMP on prolactin release and synthesis

Figs. 6 and 7 show the dose-response effect of dibutyryl cyclic AMP on prolactin release and

Vol. 162

Fig. 8. Characterization of cyclic AMP-dependent protein kinase in homogenate of GH₄C₁ cells

Phosphorylation of histone was studied by the assay system described under 'Method I' (see the Materials and Methods section) with addition of 0.2 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. (a) Protein kinase activity was measured at different concentrations of cell homogenates (given as protein concentrations). The incubation time was 10 min. The curve represents means of three different experiments. (b) Time-course of protein kinase activity with 50 µg of homogenate protein. The data are expressed as above.

synthesis respectively. The maximum biological effects were both observed at about 10 nM, and the one-half maximum effect at about 20 nM.

Activation of cyclic AMP-dependent protein kinase by thyroliberin

It is indicated that the diverse actions of cyclic AMP in animal tissues are mediated by activating tissue-specific protein kinases (Walsh *et al.*, 1968; Kuo & Greengard, 1969). The influence of thyroliberin on the activity of cyclic AMP-dependent protein kinase was therefore studied under the same experimental conditions as described for cyclic AMP formation and prolactin release.

As shown in Fig. 8, cyclic AMP-dependent protein kinase activity could be demonstrated in homogenates

Fig. 9. Activation of protein kinase in GH_4C_1 cells with thyroliberin ($3\mu M$) and by exogenous cyclic AMP (a) Suspensions of cells were preincubated with thyroliberin for the times indicated as described in the legend to Fig. 1 and in the Materials and Methods section. The supernatants were used for prolactin-release studies and the pellets homogenized and used for determination of protein kinase activity and cell protein. Protein kinase was determined by both methods I and II as described in the Materials and Methods section. Means of three different experiments that differed less than 10% are presented. (b) Protein kinase activity in cell homogenate by addition of exogenous cyclic AMP. The results are expressed as above.

of the cells. By using histone as the substrate for the enzyme, the reaction velocity was proportional to enzyme concentration (Fig. 8a) and constant with respect to time (Fig. 8b). This did occur during incubation times up to 10 min when the homogenate in the assay system contained less than $100\mu g$ of protein. Fig. 9(b) shows that one-half maximal activation of the enzyme was obtained at a concentration of $20nM$ -cyclic AMP added to the assay system. Fig. 9(a) shows the protein kinase activity after preincubation of the cells with thyroliberin ($3\mu M$). Although a slight activation did occur in the absence of thyroliberin, preincubation of the cells with the tripeptide promoted a rapid increase of the activity ratio (–cyclic AMP/+cyclic AMP) in the homogenate. After preincubation for 5 min the activity ratio approached a value of 1.0, indicating complete activation of the enzyme. By preincubation of the cells with thyroliberin for 60 min the activity ratio was slightly higher than the initial value. It is noteworthy that the time-course of activation of protein kinase by thyroliberin corresponds to the time-course of cyclic AMP formation shown in Fig. 4. No change of the total protein kinase activity in the presence of $1\mu M$ -cyclic AMP was observed (results not shown).

Discussion

The present and previous (Dannies *et al.*, 1976), investigations suggest strongly that cyclic AMP plays a role in the action mechanism of thyroliberin in prolactin-producing cells. (1) In a homogeneous cell system that synthesizes and secretes only prolactin, thyroliberin increased both hormone release and synthesis. (2) These effects can be duplicated by cyclic AMP analogues in the present and in a closely related cell strain (Dannies *et al.*, 1976). (3) Thyroliberin gave a dose- and time-dependent increase in cyclic AMP formation. (4) In a related cell strain producing both growth hormone and prolactin, phosphodiesterase inhibitors (theophylline and isobutylmethylxanthine) increased prolactin release and synthesis, and potentiated the effects of thyroliberin on cyclic AMP accumulation (Dannies *et al.*, 1976). (5) Homogenate of GH_4C_1 cells contains a cyclic AMP-dependent protein kinase with properties similar to protein kinases from other tissues (Labrie *et al.*, 1971). The natural substrate for this enzyme in the GH_4C_1 cells is unknown, but others (Jolicoeur & Labrie, 1974) have shown that chromatin, histones and acidic proteins purified from bovine anterior pituitary gland can be phosphorylated *in vitro* with homologous cyclic AMP-dependent protein kinase. (6) In experiments on intact cells, thyroliberin increased the activity ratio (–cyclic AMP/+cyclic AMP) of protein kinase, and the time-course of effect

corresponded to the increase in cyclic AMP concentrations and with initiation of prolactin release.

For these reasons we suggest that cyclic AMP concentration increases lead to protein kinase activation. These events are probably involved in prolactin release induced by thyroliberin. The interpretation of these results as they relate to the detailed mechanism of action of cyclic AMP in the GH₄C₁ cells is not yet completely understood. Different concentrations of thyroliberin caused one-half maximal effects on prolactin release and synthesis (0.25 and 4.0 nM respectively), and these concentrations were again different from that (25 nM) having one-half maximal effects on cyclic AMP accumulation and on protein kinase activation (20 nM). At present, only one type of high-affinity cell receptor ($K_d = 28$ nM) has been demonstrated (Hinkle & Tashjian, 1973). The discrepancies between the concentrations of thyroliberin causing one-half maximal stimulation of cyclic AMP formation (25 nM) and the effects on prolactin (release, 0.25 nM, and synthesis, 4 nM) may be explained if only a low percentage of the receptors needs to be occupied in order to induce the biological effects. However, when 1 mM-theophylline was present in the incubation medium of a related cell strain, thyroliberin caused one-half maximal elevations of cyclic AMP at 0.3 nM (Dannies *et al.*, 1976).

Thyroliberin has two additional effects on the GH₄C₁ cells: there is an increased uptake of ⁴⁵Ca²⁺ (J. G. Iversen & K. M. Gautvik, unpublished work), and probably as a consequence cyclic GMP concentrations are increased (K. M. Gautvik, E. Haug & M. Kriz, unpublished work). Analogues of cyclic GMP cause increased synthesis, but have no stimulatory effect on hormone release (K. M. Gautvik, E. Haug & M. Kriz, unpublished work). In addition to cyclic AMP, extracellular Ca²⁺ is probably important for the release of prolactin (Gautvik & Tashjian, 1973; Kidokoro, 1975).

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