Stimulation of Prolactin Synthesis and of Adenosine 3':5'-Cyclic Phosphate Formation by Prostaglandins and Thyroliberin in Cultured Rat Pituitary Cells

By KAARE M. GAUTVIK and MOJMIR KRIZ

Institute of Physiology, University of Oslo, Karl Johansgate 47, Oslo 1, Norway

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1. The effects of prostaglandins E_2 and $F_{2\alpha}$ on prolactin synthesis were examined in a clonal strain of rat pituitary tumour cells, and compared with those of thyroliberin. 2. The prostaglandins and thyroliberin gave a dose-related and time-dependent stimulation of prolactin synthesis. The maximal effects (about twofold increases) were observed after 54 h of treatment with 25 nM-prostaglandin E_2 and 2.5 nM-prostaglandin $F_{2\alpha}$. A similar stimulation of prolactin synthesis was observed after 250 nM-thyroliberin. The combined treatment with prostaglandins and thyroliberin did not increase prolactin synthesis over and above that obtained with each compound alone. 3. After removal of prostaglandins E_2 and $F_{2\alpha}$ there was a complete reversal of prolactin synthesis to pre-stimulation values 18 h later ($t_{1/2} \leq 9$ h). The rapid reversible effect of prostaglandins was in contrast with that of thyroliberin, where prolactin synthesis returned to control values with a $t_{1/2}$ of about 42 h. 4. Prostaglandin E_2 (5 μ M) and thyroliberin (5 μ M) increased cellular concentrations of cyclic AMP eight- and four-fold respectively. Maximal effects were observed after 2-5 min of incubation. The increases in cyclic AMP were biphasic; normal values were obtained 60 min after the start of incubation with prostaglandin E_2 or thyroliberin. 5. The dose/response curve showed that prostaglandin E_2 caused maximal increase of cyclic AMP at 50 nM. Concentrations of prostaglandin E_2 that caused half-maximal stimulation of cyclic AMP accumulation and of prolactin synthesis were 4 and 5 nM respectively. 6. Combined treatment with prostaglandin E_2 and thyroliberin in concentrations that separately caused maximal cyclic AMP increases did not result in a further increase in this cyclic nucleotide. 7. These results are consistent with a role of cyclic AMP in mediating the effects of prostaglandins and thyroliberin on prolactin synthesis. However, if cyclic AMP is involved as a common intracellular mediator of prolactin synthesis, it cannot alone explain all the effects of prostaglandins and thyroliberin in this cell system.

Prostaglandins of the E and F series have been reported to stimulate the release of several anterior pituitary hormones. Prostaglandin E_1 was thus shown to enhance the release of follicle-stimulating hormone (follicotropin), luteinizing hormone (lutropin) and growth hormone (MacLeod & Lehmyer, 1970; Orczyk & Behrman, 1972; Behrman *et al.*, 1972; Ratner *et al.*, 1974), and the E compounds were also shown to stimulate rat anterior pituitary adenylate cyclase activity (Zor *et al.*, 1969; MacLeod & Lehmyer, 1970; Deery & Howell, 1973).

The relationship between prostaglandins and prolactin is incompletely understood. MacLeod & Lehmyer (1970) found in experiments on rat pituitary glands *in vitro* that prostaglandins of the E series stimulated growth-hormone synthesis, but not prolactin synthesis. Prostaglandin E_1 when injected into the third ventricle of ovariectomized rats increased

serum prolactin concentration, but direct application to the pituitary gland did not affect serum prolactin concentrations (Harms *et al.*, 1973). However, prostaglandin $F_{2\alpha}$ injected intraperitoneally into pregnant rats or administered transcervically to pregnant women, induced an increase in serum prolactin concentration (Deis & Vermouth, 1972; Yue *et al.*, 1974). Gautvik & Kriz (1976) showed that prostaglandins of both the E and F series stimulated prolactin synthesis in cultured rat pituitary cells after a 5 h treatment period, an effect which was similar to that of thyroliberin.

In the present study the long term actions of prostaglandins on prolactin synthesis are described and are correlated with those of thyroliberin. In addition we show that both prostaglandin E_2 and thyroliberin are able to increase the intracellular concentration of cyclic AMP.

Materials and Methods

Hormones and chemicals

Rat prolactin (NIAMD-Rat prolactin-RP-1) and growth hormone (NIAMD-Rat GH-RP-1) were supplied by the National Institute of Arthritis and Metabolic Diseases, Rat Pituitary Hormone Distribution Program, Bethesda, MD 20014, U.S.A. Prostaglandins E_2 and $F_{2\alpha}$ were from the Upjohn Co., Kalamazoo, MI, U.S.A.

Culture of the GH_3 cells

The GH_3 cells that are derived from a rat pituitary tumour, produce and secrete spontaneously both prolactin and growth hormone, and the establishment and culture of these cells were described previously (Tashjian *et al.*, 1968). In the present study the cells were cultivated as monolayers in 6cm plastic tissue-culture dishes (Falcon) containing 3 ml of Hams F-10 medium (Ham, 1963) supplemented with 15% horse serum and 3% foetal calf serum (Flow Laboratories Ltd., 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 carried out with cells in the exponential phase of growth.

Radioimmunoassay of prolactin

Total extracellular amounts of prolactin were measured with a specific radioimmunoassay using the double-antibody technique (Haug & Gautvik, 1976). The smallest amount of prolactin that could be detected was 0.3 ng/ml. Before a sample of the culture medium was used in the assay, it was diluted with assay buffer (0.05 M- NaH_2PO_4 /0.1 M- $NaCl$ /0.05% NaN_3) containing 1% horse serum. Each determination was carried out in duplicate or triplicate, and each sample was examined in two separate radioimmunoassays.

Measurements of cyclic AMP

Cells were grown to late-exponential phase and scraped off the dish, centrifuged (500g, 10 min, 37°C) and resuspended in serum-free medium (Neuman-Tytell, Grand Island Biological Co., Grand Island, NY 14072, U.S.A.). Batches of the cells (100 μ l) were added to tubes containing buffer [154 mM- $NaCl$ /10 mM-Tris/HCl/1.0 mM-EDTA (sodium salt), pH 7.5], 1.5 mM- $MgCl_2$, and prostaglandins or thyroliberin at the concentrations indicated. Control tubes contained no prostaglandin or thyroliberin, 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 1 M-HCl and 10000 c.p.m. of cyclic [3H]AMP. The calculated recovery varied from

75 to 90% and the reported values were corrected for loss of cyclic AMP as observed during the extraction procedure. The tubes were kept at 4°C for 30 min and centrifuged (3000g, 30 min, 4°C). The supernatant was removed for further extraction, and the precipitate used for protein measurements after solubilization in 0.1 M- $NaOH$. The supernatants were extracted with 5 $\times$ 5 ml of water-saturated diethyl ether. The final supernatants were freeze-dried and stored at -70°C. The precipitate was dissolved in 500 μ l of 0.05 M-sodium acetate buffer, pH 6.2, and duplicate samples of 50–150 μ l were taken for radioimmunoassay. 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%. Cell protein was measured in tubes or culture dishes by the method of

Fig. 1. Effect of prostaglandin E_2 on prolactin synthesis measured as the amount of hormone accumulated in the medium after 24 and 54 h of treatment

At the start of the experiments, the monolayer GH_3 cultures were washed twice with medium followed by addition of new culture medium with and without prostaglandin. Groups of cultures were harvested at various times [(a) ▲, 54 h; △, 24 h. (b) ●, 54 h; ○, 24 h], and prolactin measured in the medium. The washed cells were used for protein determinations. For further details, see the Materials and Methods section. Results are given as mean values $\pm$ S.E.M. for four to eight dishes.

Lowry *et al.* (1951). Bovine albumin (fraction V powder; Sigma Chemical Co., St. Louis, MO 63178, U.S.A.) was used as the standard.

Results

Stimulation of prolactin synthesis by prostaglandins and thyroliberin

Measurements of the amount of prolactin that accumulates in the culture medium of GH₃ cells after a culture period of 24h or more reflect hormone synthesis for the following reasons. First, the GH₃ cells show no intracellular degradation of prolactin (Dannies & Tashjian, 1973). Second, the intracellular stores of prolactin are very small, and are equal to the amount of hormone that accumulates in the medium during 30min (Gautvik & Kriz, 1976). Third, prolactin is stable in the culture medium for the experimental period (E. Haug & K. M. Gautvik, unpublished work).

Fig. 1 shows the effect of different concentrations of prostaglandin E₂ on prolactin synthesis, measured as extracellular prolactin accumulation after 24 and 54h of treatment. There was both a time- and concentration-dependent increase in prolactin concentration. The maximum effect was obtained at 25nm-prostaglandin E₂ and half-maximal stimulation at

Fig. 3. Effect of 250nm thyroliberin on prolactin synthesis after 24 and 54h of treatment

The results are shown as mean values $\pm$ S.E.M. for four to six dishes. The amount of cell protein was not affected by the treatment and was on average 0.85mg/dish. For experimental details, see the legend to Fig. 1. $\circ$, Thyroliberin-treated; $\bullet$, control.

Fig. 2. Effect of prostaglandin F_{2α} on prolactin synthesis measured after 24 and 54h of treatment

The results are expressed as described in Fig. 1 and given as mean values $\pm$ S.E.M. for six to eight dishes. For experimental details, see the legend to Fig. 1 and the Materials and Method section. $\bullet$, 54h; $\circ$, 24h.

4nm. In addition, prostaglandin E₂ decreased the number of cells adherent to the culture dish, as shown by the decrease in measured cell protein. However, the total number of cells per dish, that is the sum of floating and adherent cells, was unchanged (results not shown).

Fig. 2 shows that prostaglandin F_{2α} has a similar effect on prolactin, the maximum increase in the hormone occurring at 2.5nm. This prostaglandin had no effect on cell adherence after 24h (results not shown), but had an effect after 54h similar to that of prostaglandin E₂, as shown in Fig. 1.

Thyroliberin was previously found to increase prolactin synthesis (Dannies & Tashjian, 1973), but on a molar basis was less effective than the prostaglandins (Gautvik & Kriz, 1976). Fig. 3 shows that 250nm-thyroliberin had a time-related effect on prolactin that resembled the maximal effect obtained with prostaglandins E₂ and F_{2α}. Since the effects of

the prostaglandins and thyroliberin were similar in certain respects, it was decided to examine the combined effects of these compounds. Table 1 shows that the stimulatory effects of prostaglandins and thyroliberin were not augmented by a combined treatment.

Effects of withdrawal of prostaglandins and thyroliberin on prolactin synthesis

Cells with increased prolactin synthesis after treatment with different concentrations of prostaglandin E_2 and $F_{2\alpha}$ for 54h showed a reversal of hormone synthesis to pre-stimulation values after 18h (Figs. 4 and 5). The absolute hormone concentrations would have been slightly different if prolactin concentrations had been expressed per mg of cell protein, but the conclusion would remain unaltered (Figs. 4 and 5). The rapidly reversible stimulation of prolactin synthesis induced by the prostaglandins was in contrast

Fig. 4. Reversal of prolactin synthesis after cessation of prostaglandin E_2 treatment

(a) Half of the cultures were treated with prostaglandin E_2 (Δ; ●, control) for 54h as described in the legend to Fig. 1. The medium from cultures was then collected for prolactin measurement, and (b) new medium containing no prostaglandin added to all the dishes for 18 and 42 h. Extracellular prolactin accumulation was measured in controls and in the groups previously treated with prostaglandin. For protein measurements the different prostaglandin-treated groups were pooled. The results are shown as mean values $\pm$ S.E.M. for four to eight samples. The shaded area represents $\pm$ twice the S.E.M. for control cultures. The concentrations of prostaglandin used in the previous treatment were: Δ, 25 nM; ●, 250 nM; ○, 2.5 nM.

Fig. 5. Reversal of prolactin synthesis after withdrawal of prostaglandin $F_{2\alpha}$ treatment

Half of the cell cultures were treated with prostaglandin $F_{2\alpha}$ for 54h at various concentrations (○, 2.5 nM; Δ, 25 nM; ●, 250 nM). The medium was collected for prolactin measurements, and the cultures then received new medium containing no prostaglandin. Extracellular prolactin concentration was determined 18 and 42h after the last medium addition. During this experiment only the highest concentration of prostaglandin $F_{2\alpha}$ decreased the amount of cell protein.

with that observed after withdrawal of thyroliberin treatment (Fig. 6). Even 42h after termination of thyroliberin treatment, prolactin synthesis remained elevated, being decreased by less than 50% of the stimulated values (Fig. 6).

Effects of prostaglandin E_2 and thyroliberin on intracellular cyclic AMP

Although the biological effects of prostaglandins and thyroliberin were similar in some respects, their stimulatory effects on prolactin synthesis had a different duration after cessation of treatment (Figs. 4, 5 and 6). It was shown (P. Dannies, K. M. Gautvik & A. H. Tashjian, Jr., unpublished work) that thyroliberin caused increases in cyclic AMP concentration in the cells and that this effect could be related to some of the biological effects of this tripeptide. Fig. 7 shows that high concentrations of thyroliberin and prostaglandin E_2 caused a temporary increase in cellular cyclic AMP, but the concentration of this cyclic nucleotide was normal at 4h, a time when the stimulatory effect on prolactin synthesis was first observed (Dannies & Tashjian, 1973; Gautvik & Kriz, 1976). Also, after 24h of treatment with prosta-

glandin E₂ and thyroliberin, no increase in cellular cyclic AMP concentration was observed (results not shown).

Fig. 6. Reversal of prolactin synthesis after withdrawal of treatment with thyroliberin

Half of the cell cultures were treated for 54h with thyroliberin (250nM) and the medium collected for hormone measurements. The cell cultures were washed once and new medium containing no thyroliberin was then added to all the cell cultures. Prolactin concentrations were measured in the medium after 18 and 42h. The amount of cell protein was not affected by thyroliberin treatment. The results are expressed as means $\pm$ S.E.M. for four to eight dishes. ○, Cultures previously treated with thyroliberin; ●, control.

Fig. 8 shows the dose/response curve of prostaglandin E₂. The lowest concentration of prostaglandin E₂ that increased the cellular cyclic AMP concentration (Fig. 8), was also able to increase prolactin synthesis (Gautvik & Kriz, 1976). Also, corresponding concentrations of prostaglandin E₂ caused half-maximal increases in both prolactin synthesis (4nM) and in cyclic AMP concentrations (5nM) (Figs. 1 and 8). These changes, however, did not occur simultaneously; increases in cyclic AMP preceded alterations in hormone synthesis. Table 2 shows the effect of combined treatment with prostaglandin E₂ and thyroliberin on cellular cyclic AMP concentrations measured after 2min of treatment. Only the combination of 500nM of both compounds resulted in an increase in cyclic AMP concentration above that observed with prostaglandin or thyroliberin alone (Table 2). These high concentrations of prostaglandin E₂ (above 500nM) could not be used for measurements of prolactin synthesis owing to toxic effects on the cells (Gautvik & Kriz, 1976). Therefore concentrations of prostaglandin E₂ and thyroliberin that separately caused maximal effects on cellular cyclic AMP and prolactin synthesis did not show an additive effect on the cyclic nucleotide concentrations (Table 2) or on hormone synthesis (Table 1).

Discussion

The modes of action of prostaglandins and thyroliberin in stimulating prolactin synthesis have certain features in common. (1) The time-courses of cyclic AMP concentration changes in response to a supra-maximal dose of prostaglandin E₂ and thyroliberin were similar. The cellular cyclic AMP concentration increased within 30s, reached a maximum after 1–5min and decreased after 1h. (2) The time between

Fig. 7. Effects of prostaglandin E₂ and thyroliberin on cellular concentrations of cyclic AMP

The results are expressed as mean values $\pm$ S.E.M. for four to six tubes, and given as pmol of cyclic AMP/tube. The average tube contained 130µg of cell protein, and the variation between each tube was less than 10%. Δ, Control; ●, prostaglandin E₂ (5µM); ○, thyroliberin (5µM).

addition of the compounds and stimulation of prolactin synthesis was also similar for prostaglandins and thyroliberin (Gautvik & Kriz, 1976).

Even if the early steps in the action of prostaglandins and thyroliberin were similar, the duration of the

effect on prolactin synthesis after withdrawal of treatment was very different. At 18h after cessation of prostaglandin treatment, prolactin synthesis had returned to normal, $t_{1/2} \leq 9$ h. Treatment with thyroliberin caused hormone synthesis to remain increased 42h after withdrawal, $t_{1/2} = 42$ h. A combination of thyroliberin and prostaglandin E_2 and $F_{2\alpha}$ in non-toxic concentrations did not increase prolactin synthesis or cyclic AMP concentrations over and above that observed when the compounds were used separately. This finding suggests that prostaglandins and thyroliberin, when used in non-toxic concentrations, may transfer their effects through the same adenylate cyclase system. However, addition of prostaglandins in high, toxic concentrations together with thyroliberin, did give an additional increase in cyclic AMP concentration above that caused by the same concentrations of prostaglandin E_2 or thyroliberin (Table 2). The explanation for this finding is not known, but stimulation of low-affinity forms of adenylate cyclases by the prostaglandin, changes in the formation of a cyclic AMP inhibitor (Ho *et al.*, 1975), or inhibition of cyclic AMP degradation, may be possible.

High concentrations of prostaglandins (above $0.5 \mu M$) were toxic to the cells and did not stimulate prolactin synthesis (Gautvik & Kriz, 1976). Also, MacLeod & Lehmeyer (1970), using prostaglandin E_1 and E_2 in μM concentrations, did not find an effect on prolactin synthesis in organ-culture experiments with the rat pituitary gland.

The possibility exists that the biological effects of prostaglandins on prolactin synthesis are mediated at least partly through activation of adenylate cyclase. The early increases in cyclic AMP, alone or in conjunction with other prostaglandin-induced intracellular changes, would result in either transcrip-

Fig. 8. Dose/response curve for the effect of prostaglandin E_2 on cellular concentrations of cyclic AMP

The experiment was carried out as described in the Materials and Methods section, and the incubation period was 2min. The results are shown as mean values $\pm$ S.E.M. for four to seven tubes, and are expressed as pmol of cyclic AMP/tube. The average protein content was $110 \mu g$ /tube.

Table 1. Effects of 3 days treatment with prostaglandin E_2 and $F_{2\alpha}$ alone or in combination with thyroliberin on extracellular prolactin accumulation

The hormone was measured as described in the Materials and Methods section, and the results expressed as means $\pm$ S.E.M. The number of dishes per group is indicated in parentheses.

Prostaglandin E_2 (nM) ...	0	25	0	0	25	0
Prostaglandin $F_{2\alpha}$ (nM) ...	0	0	25	0	0	25
Thyroliberin (nM) ...	0	0	0	250	250	250
Prolactin (μg /mg of cell protein)	16 ± 5 (4)	53 ± 11 (6)	55 ± 6 (5)	63 ± 17 (6)	62 ± 13 (6)	65 ± 8 (5)

Table 2. Effects of thyroliberin alone and in combination with prostaglandin E_2 on cellular cyclic AMP concentration

The experiments were carried out as described in the Materials and Methods section. The reaction was stopped after 2 min of incubation. The results are given as mean values $\pm$ S.E.M. for four to six tubes. The mean amount of cell protein per tube was $160 \mu g$.

Prostaglandin E_2 (nM) ...	0	0	5	50	500
Thyroliberin (nM) ...	0	500	500	500	500
Cyclic AMP concentration (pmol/tube)	1.2 ± 0.4	5.7 ± 0.4	5.3 ± 0.3	5.8 ± 0.4	15.2 ± 0.8

tional or translational events leading to increased hormone synthesis. In this respect the early effect of prostaglandins are similar to those of thyroliberin (P. Dannies, K. M. Gautvik & A. H. Tashjian, Jr., unpublished work). The GH₃ cells were shown to possess a cyclic AMP-dependent protein kinase, and cells treated with thyroliberin showed an increase of the basal activity of this enzyme (K. M. Gautvik, O. Walaas & E. Walaas, unpublished work). However, since prostaglandins and thyroliberin did not have identical biological effects in the cells (e.g. differences in the duration of action after cessation of treatment), an increase in the concentration of cyclic AMP alone cannot explain all the effects of both compounds in relation to stimulation of prolactin synthesis. In support of this assumption, thyroliberin, but not prostaglandins, have been shown to increase guanosine 3':5' cyclic monophosphate (K. M. Gautvik & M. Kriz, unpublished work). In addition the dibutyl derivatives of both cyclic AMP- and cyclic GMP-stimulated prolactin synthesis (K. M. Gautvik & M. Kriz, unpublished work). The present results suggest that prostaglandin E₂ binds to cell-surface receptors, as previously shown for thyroliberin (Hinkle & Tashjian, 1973), resulting in increased concentrations of cyclic AMP. Increasing intracellular cyclic AMP concentration is probably causally related to the action mechanism of prostaglandin and also explains some of the biological effects of thyroliberin.

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