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## CYCLIC AMP ENHANCES GENE EXPRESSION, SYNTHESIS AND RELEASE OF NEWLY SYNTHESIZED ALPHA AND LUTEINIZING HORMONE BETA SUBUNITS IN CULTURED RAT ANTERIOR PITUITARY CELLS\*

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**Abstract**—We have previously demonstrated that GnRH stimulates the synthesis of both the  $\alpha$  and LH $\beta$  polypeptide chains, an effect which was reproduced in a non additive manner by direct activation of protein kinases A and C, and abolished by actinomycin D. In the present study, we examined the effects in monolayer cultures from rat anterior pituitary cells of 8-Br-cAMP and cholera toxin, on  $\alpha$  and LH $\beta$  subunit mRNA levels and in parallel the synthesis and release of the subunits. RNA blot hybridization analysis with cDNA probes demonstrated that  $\alpha$  and LH $\beta$  mRNA levels increased by 8.9- and 4.7-fold, respectively, after a 5 h-incubation in the presence of 6 nM cholera toxin and 7.1- and 2.9-fold in the presence of 1.5 mM 8-Br-cAMP. Under the same conditions, [<sup>35</sup>S]methionine incorporation into  $\alpha$  and LH $\beta$  subunits was optimally stimulated, by 2.8-fold and 1.7 to 2.2-fold, respectively, whether the cAMP analogue 8-Br-cAMP or cholera toxin, an endogenous cAMP generator, were employed. Further, in addition to synthesis, 8-Br-cAMP appeared to increase release of neosynthesized  $\alpha$  and LH $\beta$  polypeptides, and in this respect, 8-Br-cAMP was more effective than GnRH. In contrast, 8-Br-cAMP had a weak, non significant effect compared to GnRH on the release of total radioimmunoassayable LH in the cell media. These data provide the first direct evidence for a stimulation of  $\alpha$  and LH $\beta$  gene expression by cyclic AMP, which accounts for an increase in subunit synthesis. They suggest that cAMP, as previously shown for diacylglycerols, is a potent candidate for an intracellular mediator of the GnRH effects on subunit synthesis and that it is largely responsible for sustained (protein synthesis-dependent) LH release.

Neurohormonal control of reproduction occurs in the higher vertebrates via the modulation of secretion by the hypothalamus of a decapeptide, gonadotropin-

releasing hormone (GnRH) (Kalra and Kalra, 1983). GnRH regulates in the gonadotropic cells of the anterior pituitary gland both the synthesis and the release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH). Each of these hormones is a heterodimeric glycoprotein that is comprised of two non covalently linked subunits, a common  $\alpha$  and a unique  $\beta$  subunit. It has become evident during the past few years that GnRH stimulates the synthesis of LH polypeptide chains and the accumulation of LH mRNAs (Starzec *et al.*, 1986, 1988a; Papavasiliou *et al.*, 1986; Leung *et al.*, 1987; Ramey *et al.*, 1987; Andrews *et al.*, 1988; Hamernik and Nett, 1988; Hubert *et al.*, 1988; Lalloz *et al.*, 1988a). However, the mechanisms by which the GnRH effects on synthesis are mediated within the gonadotropic cells are still unclear. Using anterior pituitary cells in culture, we have recently demonstrated that direct activation of protein kinases A and C stimulated, like GnRH, and in a non additive

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Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; cAMP, adenosine 3':5'-cyclic monophosphate; Br-cAMP, 8-bromoadenosine 3':5'-cyclic monophosphate.

manner, the synthesis of  $\alpha$  and LH $\beta$  polypeptide chains, suggesting cAMP and diacylglycerols interplay in the mediation of GnRH effects inside the cell (Starzec *et al.*, 1989). Furthermore, and consistent with this hypothesis, it has been shown that the diacylglycerol analogue 12-*O*-tetradecanoyl phorbol 13-acetate was as potent as GnRH at increasing the cell content of LH mRNAs (Andrews *et al.*, 1988; Starzec *et al.*, in preparation). In the present study, we have investigated the effects of cyclic AMP (cAMP) on  $\alpha$  and LH $\beta$  mRNA in parallel to subunit synthesis and release, using cultured rat anterior pituitary cells incubated in the presence of either the cell permeant cAMP analogue 8-Br-cAMP (Br-cAMP), or cholera toxin which generates production of cAMP in adenylate cyclase containing cells.

## EXPERIMENTAL PROCEDURES

### Materials

The materials for cell dispersion and culture were supplied either by Eurobio, Paris, France (MEM-Eagle, HAM F-10 media and foetal calf serum) or by Nunclon, Roskilde, Denmark (tissue culture dishes). [<sup>35</sup>S]Methionine (1100 Ci/mmol) was purchased from the Commissariat à l'Énergie Atomique (Saclay, France). [ $\alpha$ -<sup>32</sup>P]dCTP (400 Ci/mmol), DNA modifying enzymes, nylon filters and random priming DNA labelling kit were from Amersham (Little Chalfont, U.K.). Deoxynucleotides and 8-Br-cAMP were supplied by Boehringer (Manheim, F.R.G.) and cholera toxin by Sigma (St Louis, Mo., U.S.A.). Lipidex was from Packard (Downers Grove, Ill., U.S.A.). All other reagents were A-grade.

The rabbit antisera against reduced and carboxymethylated bovine  $\alpha$  and LH $\beta$  subunits were generously donated by Dr J. G. Pierce. The rat LH RIA kit was obtained from the National Hormone and Pituitary Program (Baltimore, Md, U.S.A.).

### Pituitary cell cultures

Anterior pituitary glands were excised from adult (9–10 week-old, 300–350 g) male Wistar rats (laboratory breeding) which had been castrated 15 days earlier, and the tissue was enzymatically dispersed using the trypsin dissociation procedure as described previously (Starzec *et al.*, 1986). The cells ( $3 \times 10^6$ ) were plated in tissue culture dishes (35 mm dia) in 150  $\mu$ l of HAM F-10 medium supplemented with 10% foetal calf serum depleted in steroids by chromatography on Lipidex. After 1.5–2 h (the time necessary for cell attachment to the surface of the dish) the culture medium volume was brought to 2 ml. The cells were cultured for 3 days at 37°C in a water and 20% O<sub>2</sub>/75% N<sub>2</sub>/5% CO<sub>2</sub> saturated atmosphere before being used in experiments.

### In situ biosynthesis

Incubation, radioactive subunit isolation and scintillation counting were essentially as previously described (Starzec *et al.*, 1986, 1988a). Briefly, proteins were labelled by incubating cells in the presence of [<sup>35</sup>S]Met (75  $\mu$ Ci/dish) in 500  $\mu$ l Krebs-Ringer medium buffered by 25 mM *N*-2-hydroxy ethyl piperazine-*N*-2-ethane-sulphonic acid (HEPES, pH 7.4) containing 0.1% bovine serum albumine,

0.2% D-glucose and 40  $\mu$ M essential amino acids minus methionine. After incubation, LH subunits were immunoprecipitated using antisera against denatured subunits. The precipitated material was purified and characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and the corresponding radioactive bands were excised from the gels, solubilized with H<sub>2</sub>O<sub>2</sub> and counted.

### RNA preparation and hybridization

RNA was extracted by lysis of incubated pituitary cells ( $3 \times 10^6$  cells/dish) in guanidine-HCl and centrifugation through CsCl according to microscale method previously described (Counis *et al.*, 1981). Total RNA was glyoxal denatured (McMasters and Carmichael, 1977), electrophoresed in 1.7% agarose and transferred onto nylon filters. Hybridizations were performed according to conventional procedures in 50% formamide, 5  $\times$  SSC (1  $\times$  SSC = 150 mM NaCl, 15 mM trisodium citrate), 5  $\times$  Denhardt's solution, 0.1% sodium sarcosinate, 50 mM phosphate buffer, pH 6.5 and 125  $\mu$ g/ml denatured salmon sperm DNA, containing either of <sup>32</sup>P-labelled  $\alpha$ , LH $\beta$  or  $\beta$ -actin cDNA probes. After overnight hybridization at 42°C, filters were washed twice at room temperature in the presence of 1  $\times$  SSC, 0.1% sarcosinate and twice in 0.1  $\times$  SSC, 0.1% sarcosinate, including one at 50°C. After autoradiography, semi-quantitative evaluation of mRNA levels was performed by densitometry.

### cDNA probes and labelling

The  $\alpha$  and LH $\beta$  rat cDNA probes are M13 recombinant subclones containing message-sense inserts which have been used as templates for cDNA synthesis with the Klenow fragment of *E. coli* DNA polymerase I and the M13 universal sequencing primer. After annealing of 2.5 ng primer to 1  $\mu$ g recombinant M13 DNA for 30 min at 65°C, the probe synthesis was achieved in 10  $\mu$ l of 30 mM Tris-acetate buffer, pH 8 containing 60 mM potassium acetate, 9.5 mM magnesium acetate, 0.5 mM dithiothreitol, 100  $\mu$ g/ml bovine serum albumin, 2.5 mM each of dATP, dTTP, dGTP, 10  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dCTP and 0.5 U Klenow enzyme. After overnight incubation at room temperature, the radioactive cDNA probe (specific radioactivity 10<sup>9</sup> cpm/ $\mu$ g) was separated from cold M13 matrix by alkaline gel electrophoresis and recovered from the gel by heat melting in the hybridization buffer.

The  $\beta$ -actin probe, used as internal standard to control  $\alpha$  and LH $\beta$  mRNA subunit measurements, was prepared by random priming of a 1150 bp mouse cDNA insert contained in a pAL41 recombinant plasmid (Alonso *et al.*, 1986). The labelling of the insert was performed according to the manufacturer's instructions (specific radioactivity 2  $\times$  10<sup>9</sup> cpm/ $\mu$ g).

### LH radioimmunoassay

LH in culture media was determined using a rat RIA kit (rat LHRP-2, NIADDK). All samples were assayed in duplicate.

### Statistical analysis

Data are presented as means  $\pm$  SE of 2–3 independent experiments performed with duplicate or triplicate cultures. Differences between control and treated groups were assessed using Student's *t*-test, *P*  $\leq$  0.05 being considered significant.

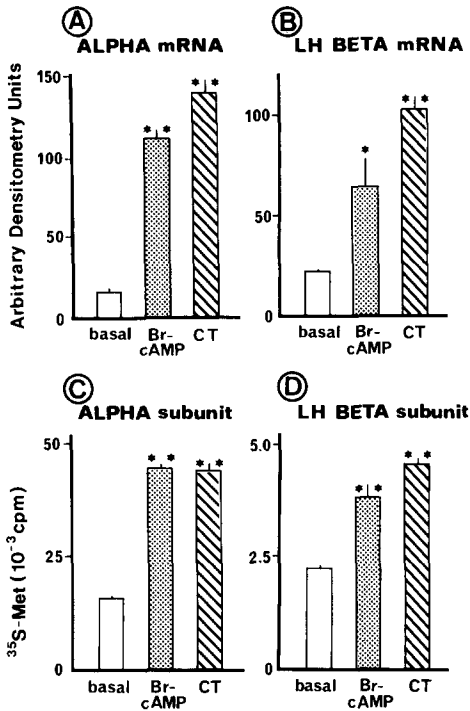


Fig. 1. Comparative effects of 8-Br-cAMP and cholera toxin on  $\alpha$  and LH $\beta$  mRNA levels and [<sup>35</sup>S]Met incorporation into polypeptide chains of  $\alpha$  and LH $\beta$  subunits. Cultured pituitary cells ( $3 \times 10^6$  per dish) were incubated for 5 h in the presence of 1.5 mM 8-Br-cAMP (Br-cAMP) or 6 nM cholera toxin (CT). The  $\alpha$  (A) and LH $\beta$  (B) mRNA were quantified in total RNA extracts by densitometry analysis of Northern blot hybridization (exposure time was 6-fold longer for LH $\beta$  than for  $\alpha$  mRNA). [<sup>35</sup>S]Methionine incorporation (cells + medium) into  $\alpha$  (C) and LH $\beta$  (D) subunits immunoprecipitated from total proteins and purified by gel electrophoresis, was determined by scintillation counting of the radioactivity. In each case, values are means  $\pm$  SE of 3 independent experiments performed in duplicate; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$  as compared to basal values.

## RESULTS

Figure 1 shows the cell content of  $\alpha$  and LH $\beta$  mRNA and the <sup>35</sup>S-labelled neosynthesized subunits after a 5 h-incubation in the presence or absence of 1.5 mM Br-cAMP or 6 nM cholera toxin. Both substances induced significant increase in  $\alpha$  [Fig. 1(A)] and LH $\beta$  [Fig. 1(B)] mRNA. Compared to basal levels,  $\alpha$  mRNA increased 7.1-fold in the presence of Br-cAMP and 8.9-fold in the presence of cholera toxin, this was a larger increase than that seen for LH $\beta$  mRNA, which increased 2.9- and 4.7-fold with Br-cAMP and cholera toxin, respectively. The differences observed in the stimulatory effect of Br-cAMP as compared to cholera toxin although visible in

Fig. 1 for  $\alpha$  and LH $\beta$  mRNA, were only significant in the case of the LH $\beta$  mRNA ( $P \leq 0.05$ ). During the same time period, [<sup>35</sup>S]Met incorporation into newly synthesized  $\alpha$  [Fig. 1(C)] and LH $\beta$  [Fig. 1(D)] was significantly enhanced 2.8-fold and 1.7 to 2.2-fold, respectively, over basal synthesis, no significant difference was observed in the respective potency of Br-cAMP or cholera toxin.

Table 1 shows a comparative study of the effects of Br-cAMP and GnRH on the synthesis of LH subunits, performed by immunoprecipitating <sup>35</sup>S-labelled  $\alpha$  and LH $\beta$  polypeptides separately from cells and media after a 5 h-incubation. As previously reported (Starzec *et al.*, 1988b), large amounts of the neosynthesized  $\alpha$  subunit was spontaneously exported into the medium in basal conditions, while the LH $\beta$  was exported only in traces. Table 1 shows that presence of 1.5 mM Br-cAMP as well as 10 nM GnRH in the cell culture stimulated the release of newly synthesized  $\alpha$  and LH $\beta$  subunits in addition to [<sup>35</sup>S]Met incorporation. Thus, in the conditions used, [<sup>35</sup>S]Met incorporation into total (cells + medium)  $\alpha$  subunit increased 2.7-fold in the presence of GnRH and 2.8-fold in the presence of Br-cAMP (no significant difference). The radioactive  $\alpha$  released into the medium from non stimulated cells (basal) represented 65% of the total neosynthesized subunit. In the presence of GnRH, 78% of the [<sup>35</sup>S] $\alpha$  was released and in the presence of Br-cAMP, 94%. In parallel, GnRH like Br-cAMP increased the amount of [<sup>35</sup>S]Met incorporated into total LH $\beta$ , by 2.1- and 2.7-fold, respectively (no significant difference). The percentage of total neosynthesized LH $\beta$  that was released in the medium was 5% in basal conditions and in response to GnRH or Br-cAMP, 14.4 and 40.4%, respectively. During the same time period, 10 nM GnRH induced a 5-fold increase in the release of radioimmunoassayable LH and 1.5 mM Br-cAMP only produced a 1.4-fold (non significant) increase.

## DISCUSSION

Since GnRH has been shown to be capable of inducing an increase in cAMP production, the role of the cyclic nucleotide within the gonadotropic cell has generated controversy especially regarding its possible involvement in the release of gonadotropins (Borgeat *et al.*, 1972; Kaneko *et al.*, 1973; Makino, 1973; Conn *et al.*, 1979; Sen and Menon, 1979; Bérault *et al.*, 1980). In the present study, we provide the first evidence that cAMP stimulates accumulation of  $\alpha$  and LH $\beta$  mRNAs in anterior pituitary cells. Indeed it is evident that the increased synthesis of  $\alpha$

Table 1. Effects of 8-Br-cAMP and GnRH on synthesis and release of neosynthesized  $\alpha$  and LH $\beta$  subunits and on release of stored LH

	Radioactivity incorporated* ( $10^{-3}$ cpm) into:				RIA-LH+ (ng/ml)
	$\alpha$		LH $\beta$		
	Cell	Medium	Cell	Medium	
Basal	8.84 $\pm$ 0.14	16.55 $\pm$ 0.31	2.38 $\pm$ 0.11	0.14 $\pm$ 0.09	45.5 $\pm$ 12.1
1.5 mM Br-cAMP	4.40 $\pm$ 0.18‡	67.95 $\pm$ 0.21‡	4.07 $\pm$ 0.13‡	2.76 $\pm$ 0.11‡	65.1 $\pm$ 5.3
10 nM GnRH	15.57 $\pm$ 0.25‡	54.41 $\pm$ 0.19‡	4.57 $\pm$ 0.15‡	0.77 $\pm$ 0.09§	229.3 $\pm$ 6.7‡

\*After a 5 h-incubation of cells in the presence of [ $^{35}$ S]Met,  $\alpha$  and LH $\beta$  polypeptides were immunoextracted separately from cells and media. The values shown are means  $\pm$  SE of 3 independent experiments.

†LH radioimmunoassayed in the medium.

Values are significantly different from control cultures: ‡ $P \leq 0.01$ ; § $P \leq 0.05$ .

and LH $\beta$  subunits is mediated by increased levels of the corresponding mRNA. In the same experimental conditions, Br-cAMP or cholera toxin had no effect on the level of  $\beta$ -actin mRNA used as reference (not shown). However, quantitative evaluation of specific mRNA levels in parallel to neosynthesized LH subunits showed that after a 5 h-incubation, the mRNA and protein levels were not increased in the same proportion. In particular, a large increase (approx. 7 to 9-fold) in  $\alpha$  mRNA was associated with a 2.8-fold increase in  $\alpha$  subunit. On the other hand, LH $\beta$  mRNA increased by approx. 3 to 5-fold, while LH $\beta$  subunit increased by 1.7 to 2.2-fold. As the concentrations of Br-cAMP and cholera toxin employed in this study caused optimal increase in LH subunit synthesis (Starzec *et al.*, 1989), it is interesting to note that there is no direct correlation between the changes in mRNA and protein subunit levels. Among several possibilities, one may suggest that only one part of the specific mRNA that accumulates is fully translatable, or that rapid and large rise in mRNA may saturate the translation machinery as observed *in vitro* (Counis *et al.*, 1982). Further investigation is necessary to clarify this point.

It is now well established that cAMP can affect the synthesis of a number of specific proteins. In the pituitary gland, prolactin (Waterman *et al.*, 1985), growth hormone (Waterman *et al.*, 1985; Barinaga *et al.*, 1985), proopiomelanocortin (Lundblad and Roberts, 1988) are regulated by cAMP as well as the  $\alpha$  and  $\beta$  subunits of the LH-related human chorionic gonadotropin (hCG) in the placenta. Using chimeric gene constructions, cAMP regulatory elements have been identified along the 5' flanking regions of the  $\alpha$  gene (Darnell and Boime, 1985) sharing with others a consensus nucleotide sequence, which can account for transcriptional control of its activity (Burnside *et al.*, 1985; Jameson *et al.*, 1986, 1988; Milsted *et al.*, 1987). The same methodology recently showed cAMP responsive regulatory domain in a 2 kb 5'

flanking region of the LH $\beta$  gene (Laloz *et al.*, 1988b). Therefore, the increased  $\alpha$  and LH $\beta$  mRNAs in cultured pituitary cells in response to Br-cAMP or cholera toxin reported in this work most likely result from increased transcription of the genes.

Although the  $\alpha$  gene is expressed in both thyrotrophs and gonadotrophs, it is clear that cAMP operates in gonadotrophs since LH $\beta$  mRNA increased. Thus, cAMP, the diacylglycerol analogue TPA and GnRH can all stimulate  $\alpha$  and LH $\beta$  mRNA accumulation, as well as polypeptide subunit synthesis. Therefore, both cAMP and diacylglycerol are good candidates as mediators of the GnRH effect on LH synthesis. Further, as they act in a non additive manner (Starzec *et al.*, 1989), the question arises of their respective roles in the control of LH gene expression.

Another point of interest to the discussion is the relationship between the regulation of synthesis and release of gonadotropins. With regard to this, diacylglycerols and protein kinase C activation may play a pivotal role in the GnRH control since they participate, in synergy with Ca $^{2+}$  and inositol triphosphate, in the LH release (Harris *et al.*, 1985; Naor and Eli, 1985; Chang *et al.*, 1988), subunit synthesis (Starzec *et al.*, 1989) and mRNA accumulation (Andrews *et al.*, 1988; Starzec *et al.*, in preparation). A role of cAMP in the rapid release of gonadotropin has been ruled out (Bérault *et al.*, 1980); our data showing only a weak effect of Br-cAMP compared to that of GnRH on LH release after 5 h-incubation are consistent with previous data from this laboratory and others (Naor *et al.*, 1978; Conn *et al.*, 1979; Sen and Menon, 1979). Contrasting with its low efficiency in inducing release of stored LH, cAMP readily stimulates, in addition to subunit production and like GnRH, release of newly synthesized  $\alpha$  and LH $\beta$  polypeptides. The method of electrophoresis employed in the study for the isolation and identification of the newly synthesized subunits did not allow us to

discriminate associated and non associated forms of LH. However, regarding the large excess of the radioactive  $\alpha$  over LH $\beta$  found in the medium whatever the experimental conditions, it appeared evident that the neosynthesized  $\alpha$  subunit is predominantly released in a free state. Moreover, the  $\alpha$  subunit isolated in the medium comprised a large proportion (approx. 50%, not shown) of a presumably *O*-glycosylated form which is unable to combine with LH $\beta$  (Parsons and Pierce, 1984; Starzec *et al.*, 1986, 1988b and unpublished data). How newly synthesized LH material is preferentially released from gonadotropic cells is to-date difficult to interpret, especially in terms of passive or active process (Kelly, 1985). In cell lines derived from placental tissue, where very little hCG exists as stored hormone, cAMP stimulation of hCG release results mostly from increased biosynthesis (Hussa, 1980; Jameson *et al.*, 1986). In the pituitary, LH released in response to GnRH has been reported to consist of two pools of the hormone: one is protein synthesis-independent and its release is rapid, the other is protein synthesis-dependent and its release is delayed, as is the accumulation of cAMP in response to GnRH stimulation (Naor *et al.*, 1978). Since cAMP has no effect on acute release of (stored) LH, we postulate according to Tang *et al.* (1984), Bourne and Baldwin (1987) and Starzec *et al.* (1988a) that the cAMP generated in gonadotrophs in response to GnRH is responsible for the sustained release of LH in terms of increased synthesis and release of the neosynthesized hormone. Thus regarding the release of stored LH and neosynthesized LH, GnRH has dual effects, while cAMP shows discrimination. Therefore these data are consistent with our hypothesis previously proposed (Starzec *et al.*, 1989), of a cascade reaction induced by GnRH stimulation of gonadotrophs, consisting of sequential activation of protein kinase C and then protein kinase A, the former being involved in acute release of LH, the latter in the synthesis of LH subunits leading to sustained release and restoration of LH stocks.

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