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Stimulation of Adenosine 3′:5′-Cyclic Monophosphate Accumulation in Anterior Pituitary Gland *In Vitro* by Synthetic Luteinizing Hormone-Releasing Hormone

(adenylate cyclase/cyclic nucleotide phosphodiesterase/rat/follicle-stimulating hormone release/adenohypophysis)

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ABSTRACT **A near-maximal dose (20 ng/ml) of synthetic luteinizing hormone(LH)-releasing hormone/follicle-stimulating hormone(FSH)-releasing hormone added to incubated anterior pituitary tissue of male rats leads to concomitant increases of intracellular concentrations of adenosine 3′:5′-monophosphate and of release of both LH and FSH. The stimulatory effect of LH-releasing hormone/FSH-releasing hormone is observed after a lag period of about 90 min and is progressive at later time intervals; a 3-fold stimulation of cAMP accumulation over control is seen after 210 min of incubation. Half-maximal stimulation of cAMP accumulation is observed between 0.1 and 1.0 ng/ml (0.1–1 nM) of LH-releasing hormone/FSH-releasing hormone. In the presence of 10 mM theophylline, the stimulatory effect of LH-releasing hormone/FSH-releasing hormone on cAMP accumulation is similar to that observed in the absence of the inhibitor of cyclic nucleotide phosphodiesterase, indicating that the releasing hormone exerts its effect by specific activation of adenylate cyclase in LH- and FSH-secreting cells rather than by inhibition of cyclic nucleotide phosphodiesterase. Since the release of growth hormone, thyrotropin, prolactin, and adrenocorticotropic hormone is not affected by LH-releasing hormone/FSH-releasing hormone, and since cAMP stimulates the release of all six adenohypophyseal hormones. the observed changes of cAMP concentrations indicate specific stimulation of adenylate cyclase activity in LH- and FSH-secreting cells of the adenohypophysis.**

Secretion of luteinizing hormone (LH) and of follicle-stimulating hormone (FSH) by the anterior pituitary gland is controlled by a neurohormone released from the hypothalamic area and carried to its specific adenohypophyseal site of action by a portal blood system (1–4). Recently, after more than 10 years of research in many laboratories, this neurohormone was isolated from porcine (5–7) and ovine (8) hypothalami and characterized as a decapeptide having the following structure: (pyro)Glu-His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (9–11). Since this peptide stimulates the release of both LH and FSH under various experimental conditions (4–8, 10–13), it has been called the LH-releasing hormone/FSH-releasing hormone (4, 5, 7) or the gonadotropin-releasing factor (12). The availability of synthetic LH-releasing hormone/FSH-releasing hormone (14–16) and of its analogs is of obvious importance for studies of the mechanism of action of this neurohormone in the adenohypophysis.

There was suggestive evidence for a role of adenosine 3′:5′-cyclic monophosphate (cAMP) as a mediator of the action of LH-releasing hormone/FSH-releasing hormone. These studies pertained to reported stimulatory effects of N^6,2′-*O*-dibutyryl cAMP and theophylline (17) and of cAMP itself (18) on LH release and of the dibutyryl derivative of cAMP on FSH release (19). Recently, a stimulatory effect of the N^6-monobutyryl derivative and of various other derivatives of cAMP on the release of both LH and FSH from rat anterior pituitaries *in vitro* has been observed (G. Chavancy and F. Labrie, unpublished). Moreover, crude hypothalamic extracts stimulate adenohypophyseal adenylate cyclase activity and concomitant release of LH (20, 21). However, definitive proof of the role of the adenylate cyclase system as mediator of the action of LH-releasing hormone/FSH-releasing hormone could be obtained only by measurements of adenohypophyseal adenylate cyclase activity or cAMP concentrations under the influence of the purified or synthetic neurohormone. This paper shows that synthetic LH-releasing hormone/FSH-releasing hormone leads to parallel stimulation of accumulation of adenohypophyseal cAMP and of release of both LH and FSH, and indicates that the neurohormone exerts its effects on cAMP by specific activation of adenylate cyclase in LH- and FSH-secreting cells rather than by inhibition of cyclic nucleotide phosphodiesterase.

Abbreviations: LH, luteinizing hormone; FSH, follicle-stimulating hormone.

MATERIALS AND METHODS

Preparation of Hemipituitaries. Anterior pituitaries from male Sprague–Dawley rats (225–250 g) were used throughout these studies. Experiments were always begun between 8:00 and 9:00 a.m. to eliminate a possible diurnal variation of the activity and sensitivity of LH- and FSH-secreting cells. After rapid removal of the posterior and intermediary lobes, the anterior pituitary was separated at the isthmus into identical halves. Each hemipituitary from the same animal was used, respectively, as control and experimental. From three to five pituitary halves were used in each group.

Incubation Procedure. Adenohypophyseal tissue was first incubated for 60 min at 37° in an atmosphere of 5% CO_2–95% O_2 in 1.0 ml of Krebs Ringer bicarbonate buffer containing 11 mM D-glucose (KRBG) as described (22). The incubation medium was then replaced by fresh buffer plus glucose and different amounts of synthetic LH-releasing hormone/FSH-releasing hormone were added for various periods of incubation as indicated in the legends to figures.

Synthesis of LH-Releasing Hormone/FSH-Releasing Hormone. The decapeptide was synthesized by the solid-phase

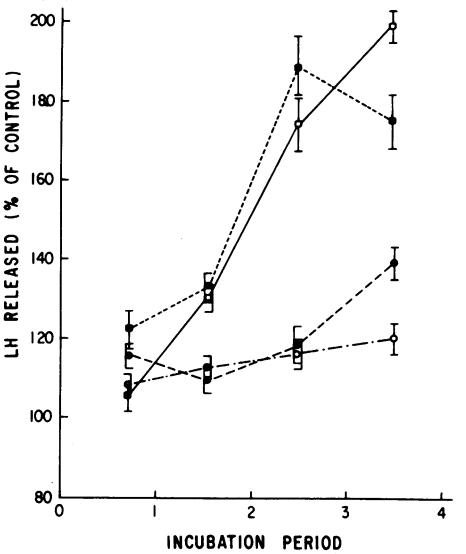

FIG. 1. Effect of dose of LH-releasing hormone/FSH-releasing hormone and time-course of incubation on the effect of the neurohormone on the release of LH from anterior pituitaries of male rats. Hemipituitaries (3 per group) were first incubated for 60 min. Incubation media were then changed every 45 min, and incubations were performed at 37° in the presence or absence of the indicated concentrations of LH-releasing hormone/FSH-releasing hormone. The amount of LH released into the incubation medium was measured by radioimmunoassay. Data are expressed as mean ± SD and as percentage of control. 0.95 ± 0.08 ng/ml of LH was released in the group containing 0.05 ng/ml of LH-releasing hormone/FSH-releasing hormone during the first 45 min of incubation. Dose of LH-releasing hormone/FSH-releasing hormone in ng/ml: ○, 0.05; ●, 0.5; □, 5; ■, 10.

method (14) and extensively purified (23). The sample used was homogeneous by thin-layer chromatography and electrophoresis and had maximal biological activity (23).

Assay of cAMP. cAMP was extracted from adenohypophyseal tissue with 5% trichloroacetic acid and measured by the receptor-binding assay of Gilman (24), with 1.0 μg of protein of the receptor preparation and 14 μg of the inhibitor per assay. Protein concentration was measured according to Lowry *et al.* (25), with bovine serum albumin as standard.

Assay of LH, FSH, Growth Hormone, Prolactin, Adrenocorticotropic Hormone, and Thyrotropin. Luteinizing hormone, follicle-stimulating hormone, growth hormone, and prolactin were measured by double-antibody radioimmunoassay (26–32). Antisera to rat LH, FSH, growth hormone, and prolactin, and pituitary hormones used for radioiodination and as standards, were kindly supplied by the National Institute of Arthritis and Metabolic Diseases, Rat Pituitary Hormone Program. Adrenocorticotropic hormone was measured by bioassay with isolated adrenal cells (33). Porcine adrenocorticotropic hormone (Mann–Schwartz) was used as standard. Growth hormone was also measured by densitometry of the proteins of the incubation medium separated by polyacrylamide gel electrophoresis (22, 34, 35). Thyrotropin was measured by bioassay (36). After appropriate corrections (37), the results were computed according to Gaddum's procedure (38) for three-point assays. Independent potency estimates obtained from four assays were tested for homogeneity (39) before calculation of weighted estimate and standard error (40) as described (41).

RESULTS

Incremental Release of LH Induced by Various Doses of LH-Releasing Hormone/FSH-Releasing Hormone. Fig. 1 shows that, under our experimental conditions, a dose of 5 or 10 ng/ml of synthetic LH-releasing hormone/FSH-releasing hormone leads to *in vitro* stimulation of LH release during the third and fourth 45-min incubation periods. A dose of 0.5 ng/ml of the neurohormone causes a slight stimulation of LH release, but the effect is observed only after a further lag period of 45 min. Similar results were obtained for FSH release (data not shown). The degree of stimulation of LH release was of similar magnitude with the two higher doses of LH-releasing hormone/FSH-releasing hormone used. Since we have observed that derivatives of cAMP lead to rapid stimulation of LH and FSH release (G. Chavancy and F. Labrie, unpublished), these data on the time-course of the effect of LH-releasing hormone/FSH-releasing hormone on LH and FSH release (Fig. 1) suggested that important changes of cAMP concentrations in LH- and FSH-secreting cells were more likely to occur after 90 min of incubation in the presence of the neurohormone. A preliminary incubation of 180 min in the presence or absence of LH-releasing hormone/FSH-releasing hormone was therefore adopted for the following experiment.

Effect of Increasing Doses of LH-Releasing Hormone/FSH-Releasing Hormone on Adenohypophyseal cAMP Concentration and LH Release. LH-releasing hormone/FSH-releasing hormone concentrations below 0.1 ng/ml do not significantly alter the concentration of adenohypophyseal cAMP (Fig. 2*A*). However, a 160% increase over control of cAMP accumulation is observed at a dose of 5 ng/ml, and a further progressive increase up to 250% (over control) is measured at a concentration of 100 ng/ml of LH-releasing hormone/FSH-releasing hormone (Fig. 2*A*). Fig. 2*B* shows that the dose–response curve of LH release to increasing concentrations of LH-releasing hormone/FSH-releasing hormone follows closely the observed changes of intracellular concentrations of cAMP. The LH-releasing hormone/FSH-releasing hormone concentration required for a half-maximal effect on both cAMP accumulation and LH and FSH release is between 0.1 and 1.0 ng/ml or between 0.1 and 1 nM.

Comparative Time-Courses of the Effects of LH-Releasing Hormone/FSH-Releasing Hormone on Adenohypophyseal cAMP Accumulation and on LH and FSH Release. Fig. 3*A* shows that in the presence of a nearly maximal dose of LH-releasing hormone/FSH-releasing hormone (20 ng/ml), the first significant rise of the cAMP concentration (50% over control) can be detected after 120 min of incubation. Longer periods of incubation in the presence of releasing hormone lead to progressive increases of the cAMP concentrations; a 4-fold stimulation over control is measured after 210 min of incubation (Fig. 3*A*). The release of both LH and FSH follows very closely the changes of cAMP concentrations, a significant increase of gonadotropin release being detected at 120 min and progressive rise of the rates of release of both hormones being measured at longer time intervals (Fig. 3*B*).

Effect of Theophylline on Stimulation of cAMP Accumulation by LH-Releasing Hormone/FSH-Releasing Hormone. When

10 mM of theophylline, an inhibitor of cyclic nucleotide phosphodiesterase, was present in both control and experimental groups (Fig. 4), the effect of LH-releasing hormone/FSH-releasing hormone on cAMP accumulation had characteristics similar to those observed in the absence of theophylline (Fig. 3A), indicating that LH-releasing hormone/FSH-releasing hormone stimulates cAMP synthesis. 10 mM of theophylline alone led to a 4-fold stimulation of cAMP accumulation during an 80-min incubation period (Fig. 4).

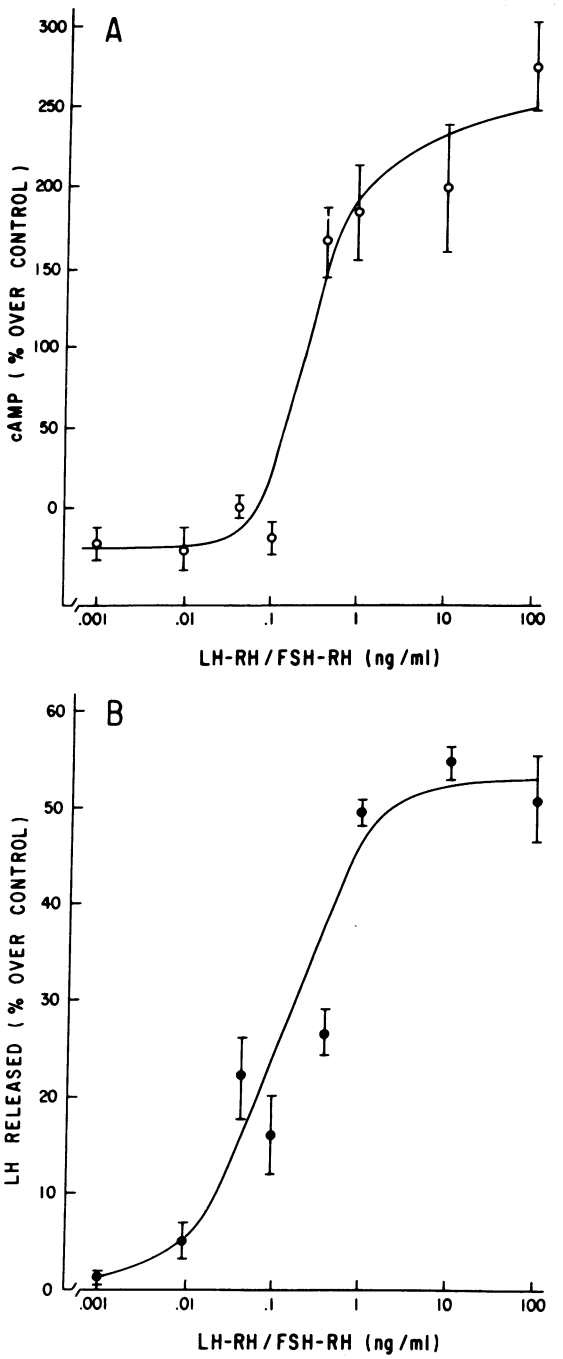

FIG. 2. Effect of increasing concentrations of LH-releasing hormone/FSH-releasing hormone on the accumulation of cAMP in adenohypophyseal tissue (*A*) and on the release of LH (*B*) from anterior pituitary tissue of male rats. Hemipituitaries (4 per group) were incubated for 60 min before addition of the indicated amounts of LH-releasing hormone/FSH-releasing hormone. The tissue was incubated for another 180 min before cAMP content and LH release were measured. Under control conditions, concentrations of intracellular cAMP and of LH released were, respectively, 8.6 ± 2.3 pmol/rat adenohypophysis and 1.04 ± 0.13 ng/ml. The percentage over control is expressed as mean ± SD (*A*): ○, control; ●, LH-releasing hormone/FSH-releasing hormone (RH) (20 ng/ml).

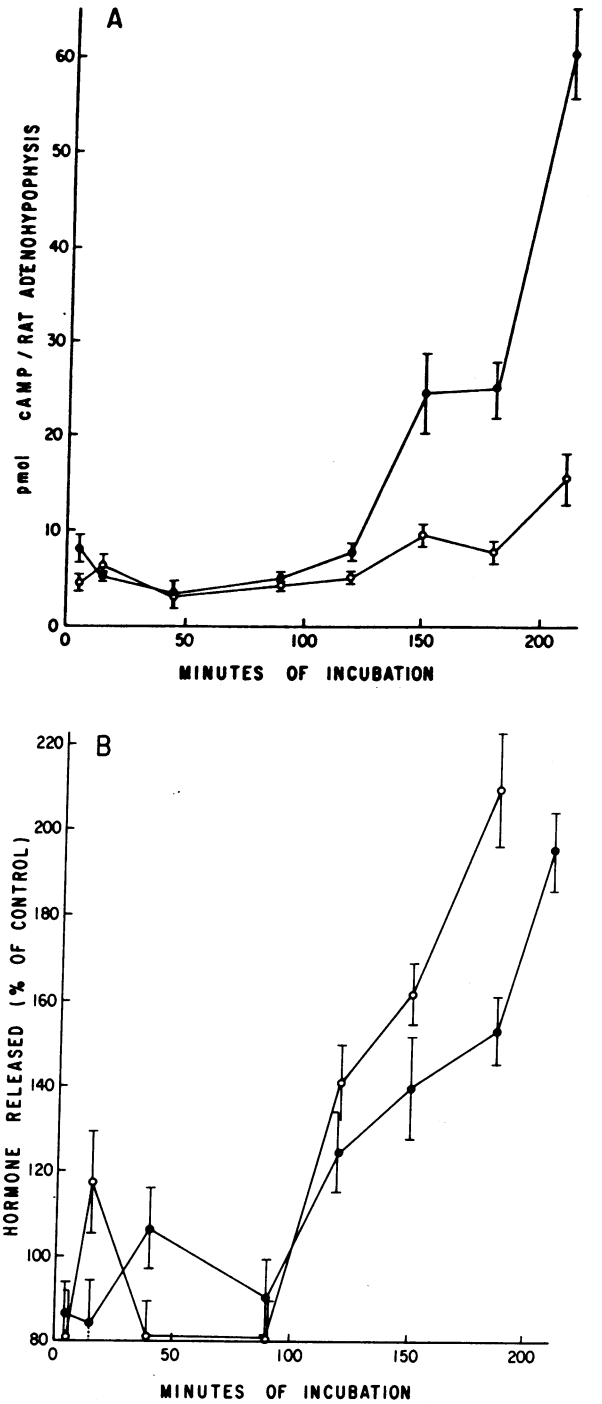

FIG. 3. Time-course of the effect of 20 ng/ml of LH-releasing hormone/FSH-releasing hormone on accumulation of adenohypophyseal cAMP (*A*) and on the release of LH (○) and FSH (●) (*B*) from anterior pituitaries of male rats. Hemipituitaries were incubated for 60 min before addition of the neurohormone, and further incubation was done for the indicated time periods.

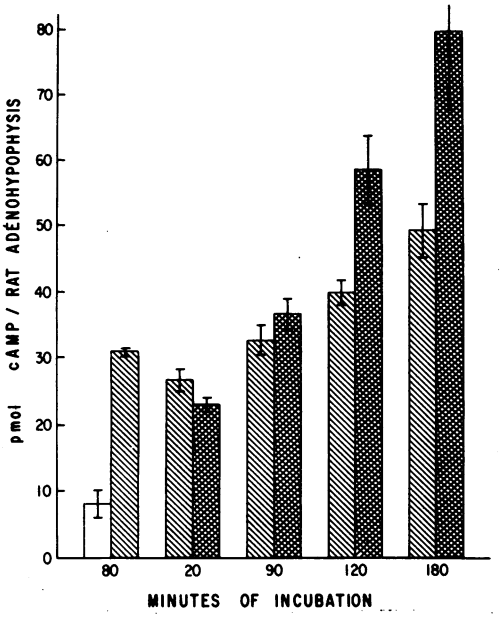

FIG. 4. Effect of LH-releasing hormone/FSH-releasing hormone (20 ng/ml) plus (*hatching*), and of 10 mM theophylline alone (*diagonal lines*), on cAMP accumulation in anterior pituitaries of male rats (*clear*, control). Except for the 80-min incubation with theophylline alone, 10 mM of theophylline was present during a 60-min prior incubation period before addition of LH-releasing hormone/FSH-releasing hormone for the indicated times of incubation. Incubations were performed as described in *Methods* and legend to Fig. 1.

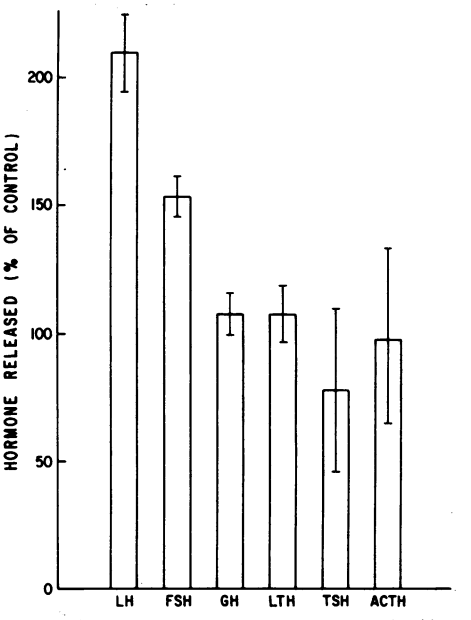

FIG. 5. Effect of LH-releasing hormone/FSH-releasing hormone (20 ng/ml) on the release of LH, FSH, growth hormone (GH), prolactin (LTH), thyrotropin (TSH), and adrenocorticotropic hormone (ACTH) by adenohypophyseal tissue of male rats. Hemipituitaries (4 per group) were incubated as described in *Methods* and legend to Fig. 1 in the presence or absence of 10 ng/ml of LH-releasing hormone/FSH-releasing hormone for 180 min. Hormonal release was determined as described in *Methods*. Data are expressed as mean ± SD.

Specificity of the Effect of LH-Releasing Hormone/FSH-Releasing Hormone on Hormonal Release. Fig. 5 shows that the stimulatory effect of synthetic LH-releasing hormone/-FSH-releasing hormone is highly specific for LH and FSH, no effect being detected on the release of adrenocorticotropic hormone, thyrotropin, growth hormone, or prolactin.

DISCUSSION

The availability of synthetic LH-releasing hormone/FSH-releasing hormone (14–16) opens new possibilities for study of the mechanism of action of this neurohormone in the adenohypophysis. Confirming previous observations made both *in vivo* and *in vitro* (4–8, 10, 13, 42–45), our data show that a single hypothalamic hormone stimulates the release of both LH and FSH. In fact, parallel release of the two gonadotropins was observed in all our *in vitro* experiments, both as a function of dose of LH-releasing hormone/FSH-releasing hormone and of time of incubation.

Although the observations of a stimulatory effect of derivatives of cAMP on the release of LH and FSH (refs. 17–19, G. Chavancy and F. Labrie, unpublished observations) suggested a role for the cyclic nucleotide on release of these two pituitary hormones, conclusive evidence for a role of the adenylate cyclase system in the action of LH-releasing hormone/FSH-releasing hormone could not be reached without measurements of adenylate cyclase activity or cAMP accumulation under the influence of the neurohormone. The present data show clearly that after 210 min of incubation in the presence of 20 ng/ml of LH-releasing hormone/FSH-releasing hormone, a 4-fold stimulation of cAMP accumulation is observed in anterior pituitary tissue, half-maximal increase of cAMP concentration being observed between 0.1 and 1.0 ng/ml (between 0.1 and 1 nM) (Figs. 2 and 3). That the cyclic cAMP accumulation is secondary to activation of adenylate cyclase activity and not dependent upon inhibition of cyclic nucleotide phosphodiesterase is indicated by the similarity of the pattern of stimulation of cAMP accumulation in the presence or absence of 10 mM theophylline (Figs. 3 and 4).

The close correlation between intracellular cAMP concentrations and rates of LH and FSH release is evidenced both as a function of dose of LH-releasing hormone/FSH-releasing hormone and time of incubation in the presence of the releasing hormone (Figs. 2*A* and 3). Since cAMP stimulates release of all six anterior pituitary hormones (17–19, 22, 35, 47–52), increased cAMP concentrations in any cell type would be expected to lead to increased release of the corresponding hormone. The absence of any effect of LH-releasing hormone/FSH-releasing hormone on the release of growth hormone, prolactin, adrenocorticotropic hormone, and thyrotropin indicates clearly that the accumulation of cAMP measured in our experiments corresponds to specific activation of adenylate cyclase activity in LH- and FSH-secreting cells. Since gonadotrophs correspond to about 7% of adenohypophyseal cells in male rats, the 3-fold stimulation of cAMP accumulation over control measured under the influence of LH-releasing hormone/FSH-releasing hormone in intact total hemipituitaries would correspond to a 45-fold specific stimulation of cAMP accumulation in gonadotrophs.

These data support the already accumulated evidence for a central role of cAMP in adenohypophyseal function (53–57) and leave little doubt about the physiological role of the adenylate cyclase system as mediator of the action of LH-

releasing hormone/FSH-releasing hormone in the adenohypophysis.

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