Vol. 87, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1979 Pages 221-228

DISSOCIATION OF CYCLIC AMP ACCUMULATION FROM THAT OF LUTEINIZING HORMONE (LH) RELEASE IN RESPONSE TO GONADOTROPIN RELEASING HORMONE (GnRH) AND CHOLERA ENTEROTOXIN*

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Received November 8, 1978

Summary: Anterior hemipituitaries from female rats were incubated in vitro in Krebs Ringer bicarbonate buffer, pH 7.2 containing 2 mg/ml of glucose in the absence and in the presence of GnRH or cholera enterotoxin. Following this incubation, the pituitaries were separated from the medium and cAMP and LH were assayed in the tissue and the medium, respectively. Incubations with GnRH in the range of 25 ng/ml to 400 ng/ml resulted in increase in LH release into the medium. Cholera enterotoxin at a concentration of 1 µg/ml, by contrast, caused no release of LH into the medium, but caused a 5-fold increase in cAMP level and this effect was concentration dependent. Cholera enterotoxin did not interfere with the GnRH-mediated LH release. It is concluded from these experiments that the ability of GnRH to increase cAMP level may be independent of its ability to release LH.

INTRODUCTION

The release of gonadotropins by the anterior pituitary is controlled by gonadotropin releasing hormone (GnRH) (1-5). Several studies have been reported in the past few years regarding the role of adenosine 3',5'-cyclic monophosphate (cAMP) as a second messenger in this process, and the outcome from these studies has been confusing. In the earlier studies, it was shown that cAMP or its dibutyryl derivative could release LH from the pituitary or from the dispersed pituitary cells in vitro (6-9). Activation of adenylate cyclase in rat pituitary in response to a crude hypothalamic extract or a purified preparation of GnRH from ovine hypothalamus has also been reported (10-12). Similarly, synthetic GnRH has also been shown to stimulate the

*Supported by NIH grant HD 07729

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0006-291X/79/050221-08$01.00/0

accumulation of cAMP in the pituitary tissue and dispersed cells (8, 13-17). By contrast, Ratner et al., (18) reported that they were unable to detect any increase in pituitary cAMP level up to 10 min after GnRH administration, but by that time the serum LH was already elevated. In addition, others have reported the absence of LH release in response to dibutyryl cAMP added to pituitary incubations (19, 20) or in response to prostaglandin E_2 (21).

In the present study we have examined the involvement of cAMP as an intermediate in LH release with the help of cholera enterotoxin, a nonhormonal peptide, which is known to stimulate cAMP accumulation in a variety of hormone responsive tissues (22, 23). Our results suggest that the ability of GnRH to stimulate cAMP production is separable from its effect on LH release from the pituitary.

MATERIALS AND METHODS

[8-^{3}H] Adenosine 3',5'-cyclic phosphate was obtained from New England Nuclear. Adenosine 3',5'-cyclic phosphate and protein kinase were purchased from Sigma Chemical Company and cholera toxin was obtained from Schwarz-Mann. LH antisera was generously donated by the National Institutes of Arthritis, Metabolism and Digestive Diseases. All other chemicals used were conventional commercial products.

Incubation procedure: Twenty-six day old female rats of Spartan strain were killed by cervical dislocation and anterior pituitaries collected in Krebs-Ringer bicarbonate (pH 7.2) containing 2 mg/ml of glucose (KRBG). The tissue was then washed twice with the same buffer. The tissue samples (4 hemipituitaries) were first preincubated at 37°C in 1 ml of KRBG for 30 min and then transferred to fresh KRBG (1 ml) containing either GnRH or cholera enterotoxin and incubated for the indicated time period at 37°C in an atmosphere of 95% O_2 and 5% CO_2. After this step, medium was saved for LH determination, and cyclic AMP and DNA were estimated in the tissue.

Radioimmunoassay of LH: Luteinizing hormone was measured in the medium at two dose levels on duplicate samples by the double antibody radioimmunoassay method (24) using rat LH preparation (NIAMD-RP-1) as a standard.

Cyclic AMP assay: Cyclic AMP content in the tissue was determined by the competitive protein binding assay as described by Gilman (25). The tissue was homogenized in 0.5 ml of 5% TCA, centrifuged and supernatant collected. To the supernatant was added 0.1 ml N HCl and 0.4 ml distilled water and it was then extracted five times with 5 ml diethyl ether saturated with water. The washed water phase was lyophilized and dissolved in 0.2 ml of 0.05 M acetate buffer (pH 4.0). Cyclic AMP concentration was measured at two dose levels according to the procedure of Gilman (23). Bound cAMP was separated from the free cAMP by filtration through Millipore filters (0.45 μM pore size) previously soaked in the same buffer and washed once with 10 ml buffer. The filters were dried, dissolved in 1 ml methyl cellosolve and counted in 10 ml of the scintillation fluid prepared by mixing 4 g PPO, 50 mg POPOP, 250 ml methyl cellosolve and 750 ml toluene.

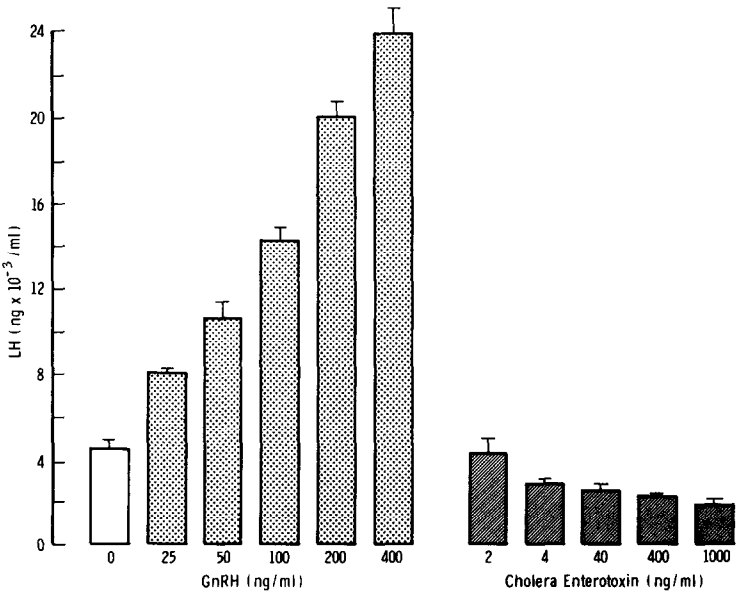

Fig. 1 Effect of increasing concentrations of GnRH and cholera enterotoxin on LH release.

Tissue (4 hemipituitaries) was incubated for 30 min at 37°C in 1 ml KRBG in an atmosphere of 95% O_2 and 5% CO_2. The medium was then replaced with fresh KRBG (1 ml) containing the reagents indicated, and incubation was continued for 2 hrs. Aliquots of the medium were analyzed for LH content by radioimmunoassay as described in the Methods section. Each value represents the mean ± S.E.M. of 4 determinations.

Determination of DNA: DNA content in the tissue was determined colorimetrically as described by Burton (26) using calf thymus DNA as standard.

RESULTS

Effect of increasing concentrations of GnRH and cholera enterotoxin on LH release: Incubation of the anterior pituitaries with increasing concentrations of GnRH resulted in an increased accumulation of LH in the medium (Fig. 1). The level of LH was higher than the base level at all doses of GnRH tested. At the 400 ng/ml concentration of GnRH, LH release was increased by about 9-10 fold. By contrast, incubation of the tissue in the presence of cholera enterotoxin caused a decrease in LH in the medium. In the presence of 1 µg/ml cholera enterotoxin, the highest concentration tested in the present studies, LH release was decreased to 60% of the control.

TABLE 1

Effect of increasing concentrations of GnRH and cholera enterotoxin on cyclic AMP accumulation in the anterior pituitary

Addition ng/ml		Cyclic AMP (pmole/µg DNA)	
		Without IBMX	With IBMX (0.5 mM)
None		0.466	1.60
GnRH	25	0.683	2.032
	50	0.755	3.048
	100	0.825	5.132
	200	0.930	5.432
	400	1.44	
Cholera enterotoxin	2	1.057	4.32
	4	1.236	5.664
	40	1.502	6.302
	400	1.978	6.802
	1000	2.434	7.835

Following 30 min preincubation at 37°C in KRBG (1 ml), the tissue(4 hemi-pituitaries) was incubated for 2 hrs with either GnRH or cholera enterotoxin in the presence or absence of 0.5 mM IBMX. Cyclic AMP content in the tissue was measured by the method as described in the Methods section. The values are the mean of duplicate determinations on duplicate samples.

Effect of increasing concentrations of GnRH and cholera enterotoxin on cAMP production in the anterior pituitary: A significant elevation of cAMP production in the tissue was observed over control at all doses of GnRH and cholera enterotoxin tested (Table 1). At a concentration of 400 ng/ml of GnRH, cAMP level increased about 3 fold over control; 1 µg/ml of cholera enterotoxin raised the level by 5 fold. Inclusion of 0.5 mM 3-isobutyl-1-methylxanthine in the incubation medium further increased the accumulation of cAMP at all concentrations of GnRH and cholera enterotoxin tested.

Time dependent effect of GnRH and cholera enterotoxin on LH release and cAMP production: The effect of GnRH and cholera enterotoxin on LH release and cyclic AMP accumulation was also tested in relation to the incubation time. As shown in Fig. 2A, the LH release in response to 100 ng/ml GnRH was increased with the increase in incubation period. The amount of LH released in the presence of cholera enterotoxin was, however, less than basal level at all time points examined. Fig. 2(B) shows the amount of cAMP produced in the

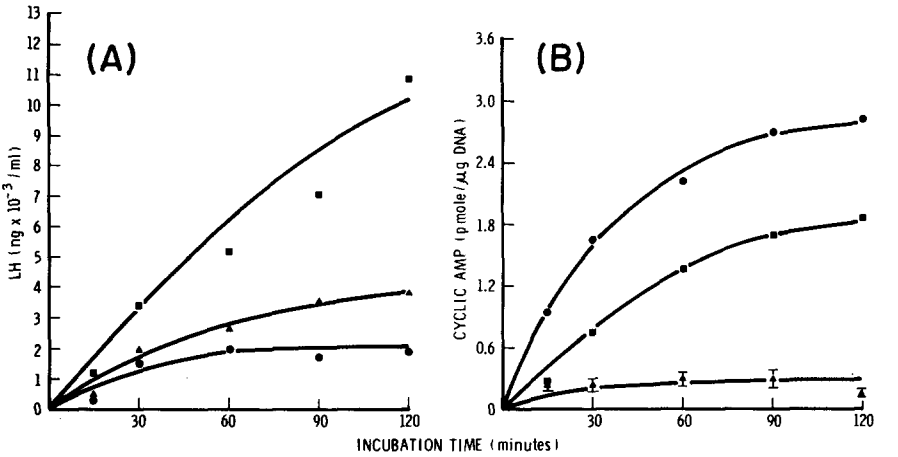

Fig. 2 Time dependent effect of GnRH (Panel A) and cholera enterotoxin (Panel B) on LH release and cyclic AMP production.

Tissue (4 hemipituitaries) was incubated for 30 min at 37°C in 1 ml KRBG in an atmosphere of 95% O_2 and 5% CO_2. The medium was then replaced with fresh KRBG (1 ml) containing either 100 ng GnRH or 1.0 µg cholera enterotoxin and incubation was carried out under similar conditions for the indicated period. Aliquots of the medium and the tissue were analyzed for LH and cyclic AMP by the methods as described in the Methods section. Each value represents the mean ± S.E.M. of 3 determinations.
▲—▲—▲ control, ●—●—● cholera enterotoxin, ■—■—■GnRH

tissue in response to GnRH and cholera enterotoxin. In both cases the amount of cAMP produced was higher than the basal level.

Effect of increasing concentrations of cholera toxin on GnRH stimulated LH release and cAMP accumulation: Since cholera enterotoxin, despite its stimulatory effect on cAMP accumulation, failed to stimulate LH release, the effect of this agent was tested on the GnRH stimulated LH release in order to examine if this agent had direct inhibitory effect on the release mechanism. The tissue was incubated with GnRH (100 ng/ml) in the absence and in the presence of increasing concentrations of cholera enterotoxin for 2 h (0.4 to 2 µg/ml) and LH was measured in the medium. At all concentrations of cholera toxin, no significant decrease in LH level was observed (Table 2).

The effect of preincubation of the tissue with different concentrations of cholera enterotoxin on its subsequent responsiveness to GnRH with respect

TABLE 2

Effect of Increasing Concentration of Cholera Enterotoxin on GnRH Stimulated LH Release in Anterior Pituitary

Treatment (ng/ml)	LH (ng/ml)
None	4949 ± 272
GnRH (100)	17183 ± 1430
Cholera enterotoxin (2000)	2815 ± 544
GnRH (100 + Cholera enterotoxin (0.4)	17138 ± 159
GnRH + Cholera enterotoxin (4.0)	14096 ± 885
GnRH + Cholera enterotoxin (40)	15027 ± 908
GnRH + Cholera enterotoxin (400)	14755 ± 885
GnRH + Cholera enterotoxin (1000)	17615 ± 2383
GnRH + Cholera enterotoxin (2000)	17388 ± 704

Tissue (4 hemipituitaries) was preincubated at 37°C for 30 min in 1 ml KRBG and then incubated in the same buffer (1 ml) for 2 hrs in the presence of either GnRH alone or cholera enterotoxin. LH was measured in the medium by radioimmunoassay as described in the Methods section. Each value represents the mean ± S.E.M. of 4 determinations.

to cAMP accumulation and LH release was examined. After the tissue was preincubated for 40 min with increasing amounts of cholera enterotoxin, GnRH (100 ng/ml) was added to the incubation medium and the incubation was carried out for a further 2 h period. LH and cAMP were then measured in the medium and tissue, respectively. As shown in Fig. 3, preincubation of the tissue with cholera enterotoxin did not alter appreciably the GnRH-stimulated release of LH. Cholera enterotoxin stimulated the accumulation of cAMP at all dose levels, but inclusion of the toxin along with GnRH failed to produce a further increase over that produced by cholera enterotoxin alone.

DISCUSSION

The present studies demonstrate that the action of GnRH on cAMP accumulation can be completely independent of its effect on LH release. This is substantiated by the fact that under experimental conditions in which cholera enterotoxin caused an increase in cAMP accumulation, no stimulatory effect was seen on LH release. The reason for this inhibitory effect is not known at the present time. Inclusion of cholera enterotoxin in the incubation medium had no effect on GnRH-mediated LH release, however. Thus, the lack of LH release

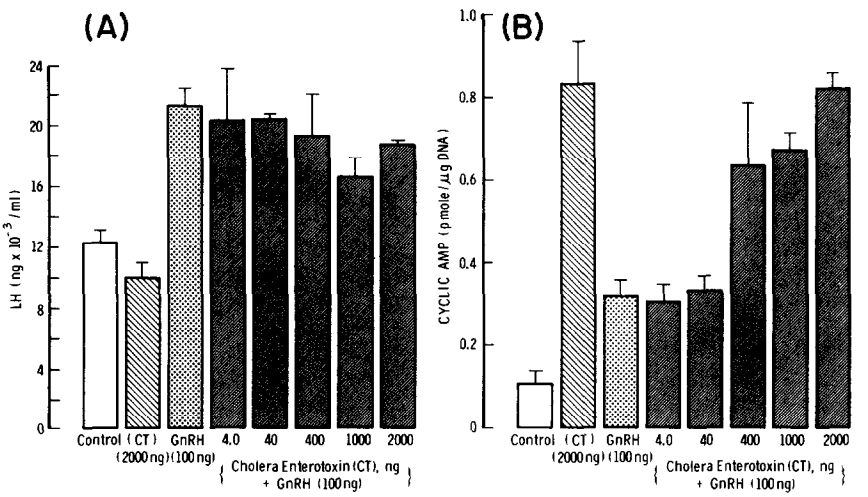

Fig. 3 Effect of preincubation with cholera enterotoxin on GnRH-stimulated LH release and cyclic AMP production.

▨ Incubation with indicated concentrations of cholera enterotoxin in the presence of 100 ng GnRH.
Panel A, LH release; Panel B, cAMP accumulation

in response to cholera enterotoxin does not appear to be due to its independent, direct effect on the LH release process. The known mechanism of action of cholera enterotoxin on responsive cell involves the interaction of the β subunit with the membrane associated GM_1 ganglioside followed by dissociation of the subunits and activation of adenylate cyclase by the α subunit (22, 23). It is unlikely that the lack of the stimulatory effect of cholera enterotoxin is due to the direct impairment of the release process. Similarly, inclusion of cholera enterotoxin in the LH-radioimmunoassay did not interfere with the assay itself. The present studies do not rule out the possibility that cAMP is an intermediate in GnRH mediated release of other pituitary hormones such as FSH as this hormone is also released in response to GnRH.

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