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DIFFERENTIAL ACTIONS OF GANGLIOSIDES ON GONADOTROPIN AND CHOLERA ENTEROTOXIN STIMULATED ADENOSINE 3':5' CYCLIC MONOPHOSPHATE DEPENDENT PROTEIN KINASE IN ISOLATED RAT OVARIAN CELLS*

Salman Azhar and K.M.J. Menon
Endocrine Laboratory, Departments of Obstetrics
and Gynecology and Biological Chemistry
The University of Michigan Medical School, Ann Arbor, Michigan 48109

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SUMMARY: Rat ovarian cells were exposed to cholera enterotoxin, and the effect on progesterone synthesis as well as on protein kinase stimulation was examined. Cholera enterotoxin stimulated ovarian steroidogenesis in a dose dependent manner similar to that of hCG. The stimulation of protein kinase by cholera toxin was followed by a lag period, whereas hCG effect was immediate. Mixed gangliosides, when added to the incubation medium, blocked the cholera enterotoxin-stimulated protein kinase activity and abolished the decrease in exogenous [^{3}H] cyclic AMP receptor activity brought about by the toxin. In contrast, under similar experimental conditions ganglioside addition elicited no effect on protein kinase activation produced by hCG or LH. The data suggest that gangliosides do not appear to be directly involved in gonadotropin binding to ovarian cell membrane and subsequent mediation of physiological response.

The available evidence suggests that adenosine 3':5' cyclic monophosphate (cyclic AMP) may be the intracellular mediator of gonadotropin mediated steroidogenesis in ovarian cells (1-6). Previous studies from this laboratory have shown that gonadotropins stimulate protein kinase in rat ovarian cells (4, 5). To evaluate further on the role of protein kinase in gonadotropin stimulated steroidogenesis, we have investigated the effects of cholera enterotoxin on protein kinase activation in rat ovarian cells. This agent was chosen because cholera enterotoxin has been shown to mimic the action of several peptide hormones in their target tissues (7, 8). Furthermore, the existence of sequence analogies between cholera enterotoxin, LH, hCG, FSH and TSH (9, 10) suggests that all these agents may act through similar mechanisms and possibly through the involvement of gangliosides. Recent studies on ganglioside inhibition of LH and hCG binding to testis plasma membrane further

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strengthen this possibility (11, 12), although no information is available on the modulation of physiological response by gangliosides in gonadal tissues.

In the present report we have examined the effect of cholera enterotoxin on protein kinase stimulation and compared the results with that obtained with hCG. Similarly, the ability of gangliosides to modulate the stimulatory effect of LH, hCG and cholera enterotoxin was also tested. Our results demonstrate that although cholera toxin can stimulate protein kinase in ovarian cells, the addition of gangliosides in the incubation medium does not affect LH and hCG stimulated protein kinase activity but does completely block cholera toxin response.

MATERIALS AND METHODS

Ovaries from twenty-six day old female (Spartan) rats were used to prepare collagenase dispersed cells as described earlier (2-5). Human chorionic gonadotropin (hCG) was a gift of the Center for Population Research, NICHD, Bethesda, MD. and provided by Dr. Robert E. Canfield of Columbia University, New York. Bovine brain mixed gangliosides and cholera enterotoxin were obtained from ICN Chemical and Radioisotope Division and from Schwarz/Mann, respectively.

Portions of cell suspension (70 µg of DNA) were incubated at 37°C in 0.4 ml of Minimum Essential Medium with Earle's salts containing 0.1% bovine serum albumin and where required 1 µg/ml of hCG, LH or cholera enterotoxin. In some cases hCG, LH or cholera toxin were preincubated with mixed brain gangliosides at 25° for 30 min before the addition of cell suspension. Final incubations were carried out at 37°C under 95% O_2 and 5% CO_2 with shaking, usually in the presence of 0.5 mM 1-methyl-3 isobutyl xanthine (MIX). Unless otherwise stated incubations with cholera enterotoxin were performed for 40 min, whereas those in the presence of hCG or LH were carried out for 10 min. After incubation the cells were homogenized in 200 ul of 10 mM Tris HCl, pH 7.2, containing 1 mM EDTA and 0.5 mM MIX. The homogenates were then centrifuged at 20,000 x g, and protein kinase was assayed by the method of Corbin et al. (13). The incubation medium (75 µl) contained 2 µmole NaF, 0.85 µmole potassium phosphate pH 6.8, 0.3 µmole $MgCl_2$, 16.5 nmole [γ-^{32}P] ATP (1.2×10^6 CPM), 750 µg mixed histone, 20-30 µg enzyme protein and, where required, 150 pmole of cyclic AMP. After incubation for 7 minutes, 50 ul of the reaction mixture was spotted on filter paper squares (Whatman 3MM, 2x2 cm) and dropped immediately in ice cold 10% trichloroacetic acid. The filters were then processed for radioactivity determinations as described by Corbin and Reimann (14). The state of activation of protein kinase was determined by measuring the activity ratio, i.e., the ratio of activity in the absence of cyclic AMP to activity in the presence of cyclic AMP (13). Values are given as the average ± S.E.M. for four determinations.

[^{3}H] Cyclic AMP binding studies were carried out by a modified procedure (15) of Gilman (16). [γ-^{32}P] ATP was prepared as described by Glynn and Chappell (17). Progesterone was measured by radioimmunoassay as described earlier from our laboratory (2).

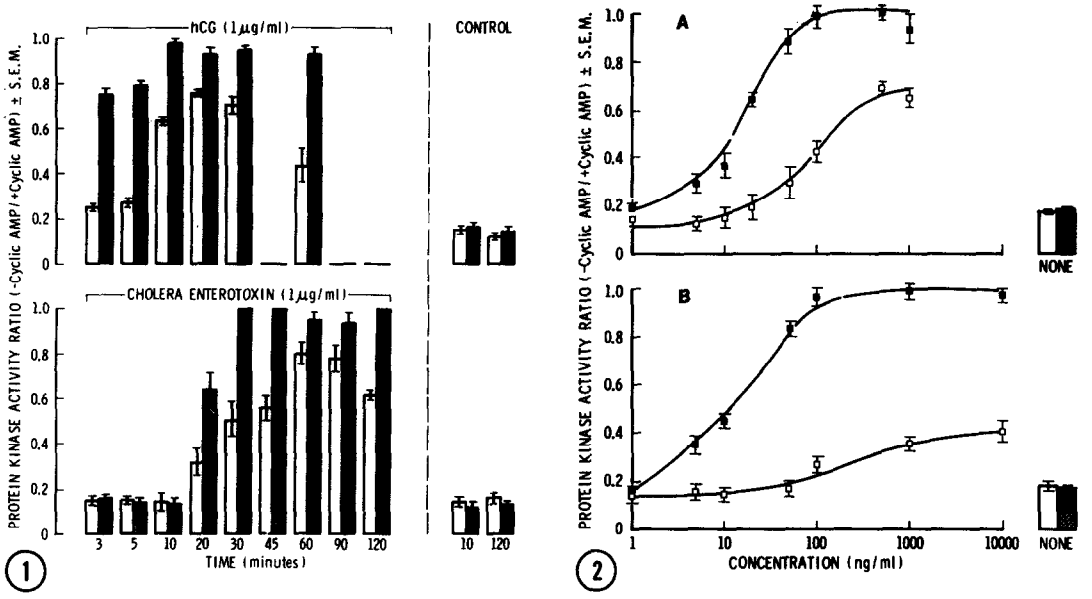

Fig. 1. Effect of incubation time on cholera enterotoxin and hCG stimulated protein kinase in rat ovarian cells.

Incubation conditions were similar to that described under "Materials and Methods". Open bar represents activity ratio in the absence of MIX; hatched bar represents activity ratio with 0.5 mM MIX.

Fig. 2. Effect of different concentrations of hCG and cholera enterotoxin on protein kinase activation.

Experimental conditions are similar to that described under "Materials and Methods". □—□—□ without MIX; ■—■—■ with 0.5 mM MIX; open bar, protein kinase activity ratio without any test substance; hatched bar,protein kinase activity ratio with 0.5 mM MIX.

RESULTS

When rat ovarian cells were exposed to cholera enterotoxin, there was a dose related increase in progesterone synthesis, the minimum concentration of toxin required was 0.1 ng/ml and maximum steroidogenesis was achieved with 100-250 ng/ml. Maximum stimulation of progesterone production in response to hCG was achieved at a final concentration of 5-10 ng/ml (data not shown).

Both cholera enterotoxin and hCG stimulated cyclic AMP dependent protein kinase activity (Fig. 1). The effect of hCG on protein kinase stimulation was

immediate and reached a maximum after 10 minutes of hormone addition in the presence of MIX (0.5 mM). In the absence of MIX, protein kinase activity ratio leveled off to a half maximum value at 60 minutes. The effect of cholera enterotoxin was of slower onset and longer duration as compared with that of hCG. The stimulation of protein kinase activity ratio measured at 30 to 45 minutes after the addition of cholera enterotoxin was comparable to that found in the cells exposed to hCG for 10 minutes.

Results presented in Fig. 2 show the effect of varying concentrations of cholera enterotoxin or hCG on protein kinase activation. Ten minutes after the addition of hCG and 40 min after the addition of toxin an increase in protein kinase activity ratio was observed within the range of doses examined. In the presence of phosphodiesterase inhibitor, protein kinase activity reached a maximum at hCG concentration of 100 ng/ml, and half maximal stimulation was attained at 12 ng/ml. In contrast, in the absence of MIX (0.5 mM), maximum protein kinase activation was achieved using an hCG concentration of 500 ng/ml, although the extent of stimulation was lower than that observed in the presence of MIX. Cholera enterotoxin stimulated protein kinase maximally at a concentration of 100 ng-500 ng/ml, and half maximum stimulation was achieved with 10 ng/ml. Unlike hCG, treatment of cells with cholera enterotoxin in the absence of phosphodiesterase inhibitor failed to stimulate protein kinase activity maximally even at a concentration of 10 μg/ml (Fig. 2).

Since gangliosides have been shown to inhibit LH and hCG binding to membrane receptors (11, 12), cholera enterotoxin binding (18), and toxin-induced lipolysis (19), we examined the possibility that the addition of gangliosides could block cholera toxin and hCG-stimulated protein kinase activity in rat ovarian cells. Results presented in Table 1 show that the addition of 10 μg/ml of mixed gangliosides completely blocked cholera enterotoxin-stimulated increase in protein kinase activity ratio and toxin-stimulated decrease in [^{3}H] cyclic AMP binding activity. In contrast, addition of 100 μg/ml or higher concentrations of mixed gangliosides did not affect either the activation of protein

TABLE 1

Effect of mixed gangliosides on the hCG and cholera enterotoxin stimulated protein kinase and cyclic AMP binding activities from rat ovarian cells.

Incubation conditions were similar to that described under Fig. 3.

Mixed Gangliosides (μg/ml)	None	hCG (1 μg.ml^{-1})	Cholera Enterotoxin (1 μg.ml^{-1})
	Protein Kinase Activity Ratio (-cyclic AMP/+cyclic AMP) ± SEM		
0	0.134 ± 0.033	1.00 ± 0	1.00 ± 0
1	0.130 ± 0.002	1.00 ± 0	0.824 ± 0.013
10	0.153 ± 0.001	0.950 ± 0.05	0.199 ± 0.001
100	0.128 ± 0.025	0.950 ± 0.08	0.168 ± 0.048
	[^{3}H] Cyclic AMP Bound (pmole.mg Protein^{-1}) ± SEM		
0	5.91 ± 0.35	2.25 ± 0.15	1.70 ± 0.34
1	5.5 ± 0.20	1.85 ± 0.18	3.73 ± 0.29
10	5.52 ± 0.80	1.83 ± 0.14	4.79 ± 0.31
100	5.85 ± 0.035	1.82 ± 0.05	5.56 ± 0.39

kinase or the decrease in [^{3}H] cyclic AMP receptor activity induced by hCG. Results presented in Fig. 3 also show that the addition of 250 μg/ml mixed gangliosides did not modulate LH stimulated protein kinase activity ratio. In contrast, gangliosides completely blocked the cholera enterotoxin stimulated protein kinase activity.

DISCUSSION

In vitro addition of cholera enterotoxin resulted in a stimulation of cyclic AMP dependent protein kinase activity in rat ovarian cells. As recent reports from a number of laboratories have indicated that most, if not all, of the biological effects of cholera enterotoxin in the intestine as well as in other organs are mediated via cyclic AMP (7, 8, 20-23), we utilized cholera toxin as a probe to investigate the role of gangliosides in relation to hCG

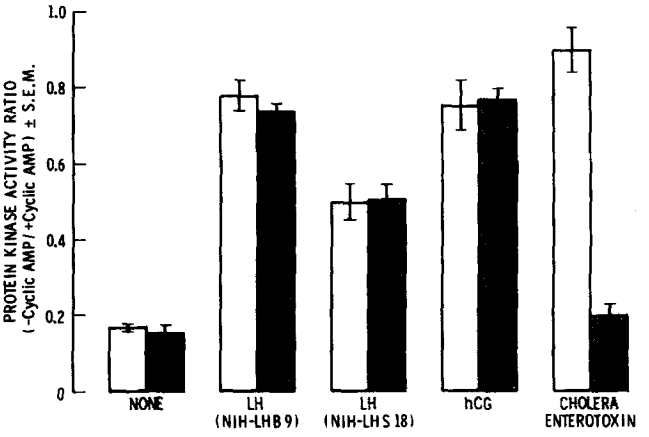

Fig. 3. Effect of mixed gangliosides on LH, hCG and cholera enterotoxin on protein kinase activation.

Incubation conditions were similar to that described under "Materials and Methods" with the exception that hormones (400 ng) were preincubated with 100 µg mixed gangliosides in a final volume of 0.3 ml. After first incubation 0.1 ml ovarian cells (75 µg DNA) were added and incubation continued for 40 min. The cells were homogenized and assayed for protein kinase activity as described under "Materials and Methods". Open bar, hormone only; hatched bar, mixed gangliosides and hormone.

induced protein kinase stimulation and steroidogenesis. In agreement with the published data on cyclic AMP production in other tissues (7, 8, 20-23), the stimulation of ovarian protein kinase by cholera enterotoxin also required a lag period (Fig. 1). Enterotoxin stimulated both steroidogenesis and protein kinase activity in a dose dependent manner similar to that observed with hCG. Since in rat ovarian cells hCG stimulates protein kinase activity by increasing the intracellular levels of cyclic AMP, the demonstration of protein kinase stimulation by cholera enterotoxin in the present report suggests that toxin mimics the hormonal response which is thought to be mediated via the adenylate cyclase-cyclic AMP system (2-5).

As has been clearly demonstrated by the experiments outlined in Table 1 and Fig. 3, in this system gangliosides did not affect LH and hCG stimulated protein kinase activity, while it completely abolished the kinase activity

stimulated by cholera toxin. These findings coupled with the failure of gangliosides to inhibit hCG stimulated steroidogenesis (24) and [^{125}I] hCG binding to rat ovarian cells (Azhar and Menon, unpublished observation) suggest that hCG exerts its effect through a separate mechanism from that of enterotoxin. In this respect our results employing the ovarian system are quite different from those in the testicular system as reported by Lee et al. (11, 12) who demonstrated that gangliosides can inhibit LH and hCG binding to testicular plasma membranes. Our results, however, suggest that the gonadotropin receptors on ovarian cells are very specific and gangliosides may not be involved in the hormone receptor interactions and the subsequent production of cyclic AMP and protein kinase activation.

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