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Receptor-Mediated Gonadotropin Action in Ovary

Differential Effects of Various Gangliosides and Cholera Enterotoxin on ^{125}I-Choriogonadotropin Binding, Production of Adenosine 3′:5′-Monophosphate and Steroidogenesis in Rat Ovarian Cells

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The effect of gangliosides and cholera enterotoxin on ^{125}I choriogonadotropin binding, cAMP formation, protein kinase activation and progesterone production have been studied in isolated rat ovarian interstitial cells. A close correlation was observed between the amount of adenosine 3′:5′-monophosphate (cAMP) produced in the cells and the extent of the activation of protein kinase in response to cholera enterotoxin and choriogonadotropin. Similarly cholera enterotoxin, like choriogonadotropin, stimulated steroidogenesis in a dose-dependent and time-dependent manner.

Addition of highly purified ganglioside mixtures abolished the stimulatory effect of cholera enterotoxin on cAMP and progesterone responses, while these agents had no effect on choriogonadotropin-stimulated responses. Similar results were also obtained when different lutropin preparations were tested. Pretreatment of ovarian cells with gangliosides did not show any enhancement in the extent of steroidgenesis stimulated by toxin or trophic hormones compared to that seen with untreated cells.

Both gangliosides and cholera enterotoxin failed to block ^{125}I-labelled choriogonadotropin binding to ovarian cells. Individual gangliosides G_{D1a}, G_{D1b}, G_{T1} and G_{M1} [designated according to Svennerholm (1963) *J. Neurochem. 10*, 613–623] did not modify gonadotropin binding to ovarian cells. Similarly gangliosides isolated from rat ovary, bovine corpus luteum and rat brain also lacked the ability to modify ^{125}I-labelled choriogonadotropin binding to cells. Analysis of ovarian cells for gangliosides indicated that major ganglioside was G_{M3} with trace amounts of other gangliosides including G_{M1} and more complex gangliosides.

The results presented in this communication suggest that gonadotropin binding and subsequent cAMP and progesterone production are not influenced directly by gangliosides. In contrast, choleragen-stimulated cellular events in the ovarian cells were completely inhibited by gangliosides. This suggests that these two agents exert their effects on the responsive system by interacting through separate components on the cell surface.

The mechanisms by which gonadotropins (lutropin and choriogonadotropin) regulate steroidgenesis in the ovary are not fully understood. Gonadotropin receptors located in the plasma membrane of target cells are now recognized as the specific initial mediators through which gonadotropins influence cellular events including steroidogenesis [1–19]. The available evidence suggests that the initial binding of hormone to cell surface receptor leads to the activation of membrane-bound adenylate cyclase, accumulation of cAMP, activation of cAMP dependent protein kinase(s) and subsequent stimulation of steroidogenesis [1–10, 20–28]. However, little is known about the involvement and role of various membrane components in receptor-mediated gonadotropin action including regulation of cAMP and steroid responses [1–10, 20–28].

Cholera enterotoxin (choleragen), a protein of M_r about 84000 [29] secreted by *Vibrio cholera*, has been shown to stimulate adenylate cyclase in a

Abbreviations. cAMP, adenosine 3′:5′-monophosphate; G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{M3}, *N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{M3}, *N*-acetylneuraminyl-galactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{D1b}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl-*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{T1}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl-*N*-acetylneuraminyl)-galactosylceramide. Gangliosides are designated according to Svennerholm [78].

variety of mammalian tissues [30, 31] and in some cases increases the responsiveness of adenylate cyclase to hormonal stimulation. The initial event in the action of choleragen involves a high-affinity binding to cell surface receptor sites [32–37] believed to be ganglioside G_{M1} [32–34, 36–49]. Ganglioside G_{M1} has been shown to block the biological effects of the toxin in several systems [32–50]. Recently, comparison of a partial sequence [51] and the complete sequence [52] of the β chain of the cholera enterotoxin with the β chain of the glycoprotein hormones such as human choriogonadotropin, lutropin, follitropin, and thyrotropin, revealed that a segment comprising the first 40 residues of the toxin is chemically similar to an internal segment of the hormones. The molecular weights of the β chain of the glycoprotein hormones and the β chain of cholera toxin are similar. Furthermore, like the cholera enterotoxin receptor [32–34, 36–49], it was postulated that ganglioside or a ganglioside-like structure may be an integral part of glycoprotein hormone receptors [53–58]. More recently gangliosides have been shown to interact with thyrotropin, lutropin and human choriogonadotropin and in turn block their binding to respective receptors [54, 56–58]. While these findings provide strong evidence for the idea that the gangliosides may be part of glycoprotein hormone receptors, we are unaware of any evidence which suggests that gangliosides modify biological responses elicited by these hormones.

Previous studies from this laboratory have shown that collagenase-dispersed rat ovarian interstitial cells respond to gonadotropin with increases in cAMP accumulation [7, 9, 23, 25–27], protein kinase activity [22, 23, 25–27] and steroidogenesis [7, 9, 26, 28]. In the present paper we have investigated in this system the effect of ovarian gangliosides and gangliosides from other sources on ^{125}I-labelled choriogonadotropin binding and the effect of these agents on subsequent gonadotropin-stimulated cAMP and progesterone responses.

EXPERIMENTAL PROCEDURE

Materials

Cholera enterotoxin was purchased from Schwarz/Mann (Orangeburg, N.Y., U.S.A.). Highly purified bovine brain gangliosides were purchased from ICN Pharmaceuticals, (Irvine, Calif., U.S.A.). Individual gangliosides were isolated by preparative thin-layer chromatography of commercially purchased gangliosides. Precoated Silica Gel G thin-layer plates 0.25 mm (20 × 20 cm) were obtained from Analtech Inc. (Newark, Del., U.S.A.). Precoated silica gel 60 F_{254} thin-layer plates were purchased from Brinkman Instrument Inc. (DesPlaines, Ill., U.S.A.). Unisil and DEAE-Sephadex A-25 (40 to 120 μm) were supplied by Clarkson Laboratories (Williamsport, Pa., U.S.A.), and Pharmacia Fine Chemicals (Piscataway, N.J., U.S.A.), respectively. Purified human chorionic gonadotropin (11 500 U/mg) was obtained from Dr R. E. Canfield (Columbia University Collge of Physicans and Surgeons, New York, N.Y., U.S.A.) through the Center for Population Research, National Institutes of Child Health and Human Development, Bethesda, Md., U.S.A. Ovine lutropin (NIH-LH S18) and bovine lutropin (NIH-LH-B9) were gifts of Hormone Distribution Program, National Institutes of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md., U.S.A. All other chemicals used were of reagent grade. Eagle's minimum essential medium with Earle's salts (medium 109) was purchased from Grand Island Biological Company (Grand Island, N.Y., U.S.A.). ^{125}I-labelled choriogonatotropin was prepared as described earlier [9, 18, 19].

Preparation of Rat Ovarian Cells

Ovarian interstitial cells were prepared from 25-day-old rat ovaries (Sprague Dawley) by the procedure described earlier [7, 9].

Incubation Conditions for Choriogonadotropin, Lutropin and Cholera Enterotoxin Stimulation of Progesterone Production by Ovarian Cells

Unless otherwise stated, cells ($2.5-4 \times 10^6$ cells) were incubated in a final volume of 0.4 ml of medium 109 containing 0.1% bovine serum albumin (w/v) in the presence and absence of added hormone. Incubations were carried out at 37 °C in an atmosphere of O_2/CO_2 (95/5%, v/v) usually for 3 h. Other details, if any, are given under individual tables and figures.

Radioimmunoassay for Progesterone

Following incubation as described above, the sample tubes were placed in a boiling water bath for 3 min and 0.6 ml water and 10 μl (≈ 10000 counts/min) of [1,2-^{3}H]progesterone to monitor recovery were added and the samples left in the cold overnight. After 12 h, samples were extracted with light petroleum and assayed for progesterone by radioimmunoassay as described earlier [7, 9].

Gonadotropin and Cholera Enterotoxin Stimulation of cAMP

Cells ($2.5-4 \times 10^6$ cells) were incubated in a final volume of 0.4 ml of medium 109 containing 0.1% bovine serum albumin (w/v), in the presence or absence of 0.5 mM 3-isobutyl-1-methylxanthine and where required choriogonadotropin, lutropin or cholera enterotoxin were also added. After incubation at 37 °C for 60 min in the presence of O_2/CO_2 (95/5%,

v/v) cells and medium were processed and assayed for cAMP according to Gilman [59] as described previously [25].

Activation of cAMP-Dependent Protein Kinases by Gonadotropins and Cholera Enterotoxin

Cell suspension (1.2×10^7 cells) in 0.4 ml of medium 109 containing 0.1% bovine serum albumin (w/v) were incubated with or without test substance at 37 °C in the presence of O_2/CO_2 (95/5%, v/v). After incubation for 40 min the cells were centrifuged ($400 \times g$, 5 min) and homogenized in 200 µl of 10 mM Tris-HCl buffer pH 7.2; containing 1 mM EDTA and 0.5 mM 3-isobutyl-1-methylxanthine. Following centrifugation at $20000 \times g$ for 20 min in the cold (4 °C), the clear supernatant was used to assay protein kinase activity by the method of Corbin et al. [60]. The incubation mixture contained in a final volume of 75 µl, 12 mM potassium phosphate pH 6.8, 20 mM NaF, 4 mM $MgCl_2$, 0.22 mM [γ-^{32}P]ATP (90000 to 100000 counts $\times$ min^{-1} $\times$ mmol^{-1}), 10 mg/ml of mixed histone, cellular extract and where required 2 µM cAMP. In each case the reaction was stopped after 5 min of incubation at 30 °C by spotting 50 µl of the reaction mixture on Whatman 3 MM filter paper disc and immersing in ice-cold 10% trichloroacetic acid as described by Corbin and Reiman [61] with the modification that disc were washed four times with 10% trichloroacetic acid with at least one overnight wash [27]. They were then washed for 30 min in ethanol and 30 min in diethyl ether before drying and counting in a Beckman LS230 liquid scintillation spectrophotometer in vials containing 10 ml of a solution of 4 g of Omniflour/l toluene. Protein kinase activity was expressed as the activity ratio, i.e. the ratio of activity in the absence of cAMP to that in the presence of 2 µM cAMP [60].

Choriogonadotropin Binding to Ovarian Cells

The method used for the binding assay of ^{125}I-labelled choriogonadotropin to intact interstitial cells was identical to that described by Clark and Menon [9].

Gangliosides Isolation, Purification and Characterization

Ovarian gangliosides were isolated and purified using the procedure described by Ledeen et al. [62]. Ovarian cells equivalent to 1 g ovaries were homogenized in 20 ml of chloroform/methanol (1/1, v/v) and the suspension was centrifuged at $1000 \times g$ for 30 min. The supernatant was saved, and the pellet was reextracted twice with 10 volumes of the same solvent. The combined supernatants were diluted to 200 ml giving an adjusted composition of chloroform/methanol/water (30/60/8, v/v/v).

The entire 200 ml sample in chloroform/methanol/water (30/60/8, v/v/v) was applied to a previously equilibrated DEAE-Sephadex column at a rate of approximately 1 ml/min according to the procedure of Ledeen et al. [62]. The first fraction (I) consisted of 300 ml chloroform/methanol/water (30/60/8, v/v/v) (sample volume + 100 ml), and the second fraction (II) was eluted with 180 ml of chloroform/methanol/0.8 M sodium acetate (30/60/8, v/v/v). Fraction II was evaporated to dryness and the residue was resuspended in 16 ml of 0.1 M NaOH in methanol and incubated at 40 °C for 3 h. The reaction mixture was acidified to pH 2 by dropwise addition of 1 M HCl in an ice bath, and after the addition of 1 ml of 0.5 M EDTA, the mixture was dialyzed against distilled water for 2 days. The dialyzed material was lyophilized, and the resulting residue was suspended in 20 ml of chloroform/methanol (1/1, v/v) and solubilized with mild sonication. Following centrifugation the filtrates were evaporated to dryness. The dried material was suspended in 4 ml of chloroform/methanol (1/1, v/v); 6 ml of chloroform was then added and the mixture was applied to a Unisil (2 g) column packed and equilibrated with chloroform. The column was first eluted with 140 ml of chloroform/methanol (80/20, v/v) at a flow rate of 0.5 ml/min. The adsorbed gangliosides were next eluted with 300 ml of chloroform/methanol (1/1, v/v). The ganglioside fraction was evaporated to dryness. The dry material was dissolved in chloroform/methanol (2/1, v/v), sonicated, and cleared by centrifugation. The quantitative and qualitative determinations of ganglioside *N*-acetylneuraminic acid were performed on this supernatant.

Two samples of about 10–20 µg of *N*-acetylneuraminic acid were taken and the quantity of sialic acid was determined by the resorcinol method of Svennerholm [64], using butanol/butylacetate (15/85, v/v) to extract the chromophore [65]. The color was read at 580 nm in a Gilford spectrophotometer using free sialic acid as standard.

Thin-layer chromatography was carried out on precoated plates of silica gel G (Analtech Inc.) or silica gel F_{254} plates (Brinkman Inc.) (20×20 cm); 10 to 12 µg of total *N*-acetylneuraminic acid and about 10 µg reference standards were applied. The solvent mixtures used [66] were chloroform/methanol/water (60/35/8, v/v/v) containing 20 mg $CaCl_2 \cdot 2\,H_2O$ per 100 ml solvent and chloroform/methanol/2.5 M NH_4OH (60/35/8, v/v/v) containing 20 mg KCl per 100 ml of solvent. Chromatography was performed for 2.5 h twice in a tank at room temperature (25 °C). Gangliosides were localized by resorcinol spray [67]. For the quantitative determination of individual gangliosides, the bands were visualized by brief exposure to iodine vapors. After iodine sublimation,

Table 1. *Effect of cholera enterotoxin and choriogonadotropin on cAMP accumulation and protein kinase activation in isolated rat interstitial cells*
Ovarian cells (3×10^6 cells for cAMP or 1.3×10^7 cells for protein kinase) were incubated in a final volume of 0.4 ml medium 109 containing 0.1% bovine serum albumin and indicated concentrations of choriogonadotropin or cholera enterotoxin in the presence or absence of 0.5 mM 3-isobutyl-1-methylxanthine. Following incubation for 60 min in the case of cAMP and 40 min for protein kinase in the presence of O_2/CO_2 (95/5%) at 37 °C the tubes were processed for respective assays as discussed under Experimental Procedure. Results are mean of triplicate samples on duplicate determinations ± S.E.M.

Additions	cAMP	Protein kinase (− cAMP/+ cAMP)
	pmol · μg DNA^{-1}	
None	0.55 ± 0.05	0.162 ± 0.029
3-Isobutyl-1-methyl-xanthine (0.5 mM)	0.96 ± 0.02	0.209 ± 0.027
Choriogonadotropin (1 μg/ml)	3.60 ± 0.22	0.462 ± 0.086
Cholera enterotoxin (1 μg/ml)	2.93 ± 0.26	0.483 ± 0.030
Choriogonadotropin (1 μg/ml) + 3- Isobutyl-1-methylxanthine (0.5 mM)	12.52 ± 0.51	0.805 ± 0.04
Cholera enterotoxin (1 μg/ml) + 3-Isobutyl-1-methylxanthine (0.5 mM)	14.92 ± 0.38	0.937 ± 0.035

marked areas of plates were scraped, eluted as described by Ledeen [62], and the sialic acid contents determined spectrophotometrically [64,65].

RESULTS

Effect of Cholera Enterotoxin on cAMP Accumulation and Protein Kinase Activation and Steroidogenesis in Rat Ovarian Cells

Incubation of ovarian cells with choleragen or choriogonadotropin produced an increase in their cAMP content (Table 1), and addition of 0.5 mM 3-isobutyl-1-methylxanthine potentiated the action of both toxin and hormone. Similarly, cells incubated with 1 μg/ml of either cholera enterotoxin or choriogonadotropin increased protein kinase activity ratio (−cAMP/+cAMP), and as in the case of cAMP, addition of phosphodiesterase inhibitor potentiated the action of both these agents (Table 1). Since in rat ovarian cells gonadotropin stimulates protein kinase activity by increasing the intracellular levels of cAMP [22,23,25], the demonstration of protein kinase stimulation by choleragen suggests that the toxin mimics the hormonal response which is thought to be mediated via the adenylate cyclase-cAMP system [7,9]. Cholera enterotoxin also stimulated progesterone production in a dose-dependent manner. Although choriogonadotropin stimulated a maximum amount of progesterone production after 2 h of incubation, a 3 h incubation

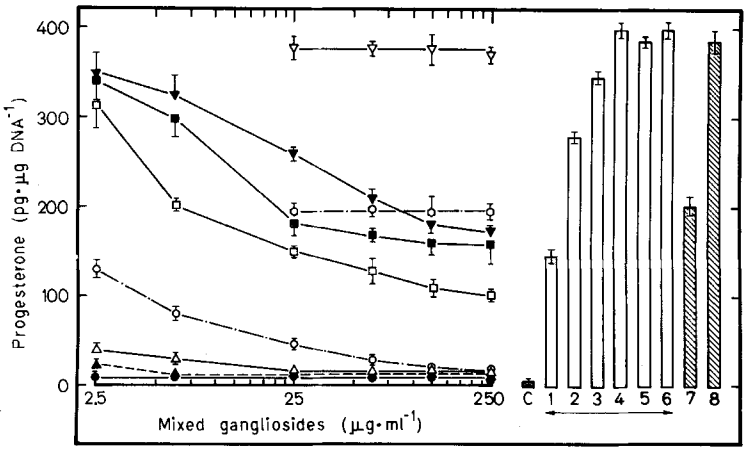

Fig. 1. *Action of exogenously added mixed gangliosides on progesterone synthesis by ovarian cells stimulated by cholera enterotoxin and choriogonadotropin.* Different concentrations of cholera enterotoxin or choriogonadotropin were incubated with bovine brain mixed gangliosides in medium 109 containing 0.1% bovine serum albumin in a volume of 0.3 ml at 35 °C for 30 min. Following preincubation 0.1 ml ovarian cells (3×10^6 cells) were added and incubation continued for an additional 3 h at 37 °C in an atmosphere of O_2/CO_2 (95/5%, v/v). Following incubation, samples were extracted and assayed for progesterone by radioimmunoassay as described in Experimental Procedure. The concentrations of various agents shown in the figure were the final concentrations after the addition of cells. (●——●) No addition; (▲——▲) cholera enterotoxin (2.5 ng/ml); (△——△) cholera enterotoxin (10 ng/ml); (○- · -○) cholera enterotoxin (100 ng/ml); (□——□) cholera enterotoxin (1μg/ml); (■——■) cholera enterotoxin (2.5 μg/ml); (▼——▼) cholera enterotoxin (10 μg/ml); (◇ ——◇) choriogonadotropin (1 ng/ml); (▽——▽) choriogonadotropin (10 ng/ml). Bar C represents progesterone production without any test substance. Bars 1 to 6 represent progesterone production in the ovarian cells in response to 2.5, 10, 100, 2500, 10000 ng/ml of cholera enterotoxin without added gangliosides. Hatched bars 7 and 8 represent progesterone production elicited by 1 and 10 ng/ml of choriogonadotropin. Results are the mean of triplicate samples on duplicate determinations ± S.E.M.

period was required to reach maximum steroid production in response to choleragen (data not shown). Results presented so far suggest that choriogonadotropin and cholera enterotoxin effectively and almost equally stimulate cAMP and progesterone responses in ovarian cells. However, it is important to mention here that since these cells were treated with collagenase and may therefore not entirely represent the situation *in vivo*, further experiments reported below deal with effect of gangliosides on these two responses as well as on the binding of ^{125}I-labelled choriogonado ropin to ovarian cells.

Effect of Exogenous Gangliosides on Progesterone Production and cAMP Formation Stimulated by Cholera Enterotoxin and Gonadotropin

Since gangliosides have been previously reported to block binding of thyrotropin, lutropin, and choriogonadotropin to their receptors [54, 56–58] and also to modify cholera enterotoxin responses in other biological systems [33–47, 50], we tested the possibility that gonadotropin-ganglioside or cholera enterotoxin-ganglioside interactions could lead to subsequent modification of progesterone and cAMP responses. Results presented in Fig. 1 show that incubation of cholera enterotoxin with gangliosides blocked the steroidogenic effect of choleragen in a dose-dependent manner. Addition of 25 μg/ml mixed gangliosides completely blocked stimulation of steroidogenesis by 1 ng or 10 ng/ml of enterotoxin. Similarly, higher concentrations of gangliosides effectively blocked progesterone production promoted by higher concentrations of cholera enterotoxin (Fig. 1). On the other hand, the steroidogenic response to choriogonadotropin was unaffected by the same concentrations of gangliosides. Similar results were also obtained when different preparations of lutropin were tested as stimulators of steroidogenesis in rat ovarian cells. As in the case of choriogonadotropin, different concentrations of gangliosides failed to modulate lutropin-stimulated steroidogenesis (Table 2).

The results presented in Table 3 also show that gangliosides completely blocked the increase in cAMP levels caused by cholera enterotoxin. However, gangliosides were completely ineffective against gonadotropin-stimulated cAMP production (Table 3). Similarly, we could not detect any change in ^{125}I-labelled choriogonadotropin binding to rat ovarian cells in the presence of gangliosides. Pretreatment of ^{125}I-labelled choriogonadotropin with different concentrations of mixed bovine brain gangliosides (up to 500 μg/ml) did not affect subsequent binding of the hormone to the cells (Fig. 2). Similarly, cholera enterotoxin did not compete with gonadotropin binding, while under identical experimental conditions, 250 ng/ml unlabelled choriogonadotropin completely blocked the binding of ^{125}I-labelled choriogonadotropin to ovarian cells (Fig. 2).

Table 2. *Effect of mixed gangliosides on gonadotropins and cholera enterotoxin stimulation progesterone synthesis by rat ovarian cells*
Incubation conditions were the same as described in Fig. 1. Results are ± S.E.M.

Additions	Bovine brain mixed gangliosides (μg/ml)			
	0	2.5	25	100
	pg progesterone/μg DNA			
None	4.8± 1.0	6.1± 2	5.2± 1.3	5.0± 0.9
Lutropin (NIH-LH-S18) 50 ng/ml	121 ±10	132 ± 6	118 ±20	138 ±18
Lutropin (NIH-LH-B9) 50 ng/ml	193 ±12	179 ±14	189 ±17	195 ±24
Chorio-gonado-tropin 2.5 ng/ml	238 ± 9	214 ±15	231 ± 6	214 ±17
Cholera entero-toxin 100 ng/ml	201 ±10	48 ± 6	13 ± 5	7.3± 1.6

Lack of Effect of Different Gangliosides and Mixed Gangliosides of Different Composition on ^{125}I-labelled Choriogonadotropin Binding to Ovarian Cells

Since in Fig. 2 we used only one type of mixed gangliosides (bovine), in subsequent experiments we also tested the effect of gangliosides of variable composition on ^{125}I-labelled choriogonadotropin binding to ovarian cells. Pretreatment of choriogonadotropin or ovarian cells with gangliosides from rat ovary or from bovine brain also could not produce any change in the ^{125}I-labelled choriogonadotropin binding activity. Further chromatographically pure individual gangliosides G_{T1}, G_{M1}, G_{D1a} and G_{D1b} up to 100 μg/ml also failed to modulate gonadotropin receptor interaction on ovarian cells. Similarly, mixed gangliosides isolated from rat ovary, bovine corpus luteum and rat brain did not affect gonadotropin binding to cells (Table 4).

The results presented in Fig. 3 show typical thin-layer chromatographic analyses of the gangliosides obtained from ovarian cells. The major ganglioside found in the intact cells was identified as ganglioside

Table 3. *Effect of mixed gangliosides on cAMP synthesis by rat ovarian cells stimulated by choriogonadotropin, lutropin and cholera enterotoxin*

Cells (4×10^6 cells) were incubated in a final volume of 0.4 ml of medium 109 containing 0.1% bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine, indicated concentrations of gonadotropins or cholera enterotoxin, and where required, indicated concentrations of gangliosides were also added. Before addition of cells, hormone and gangliosides were preincubated at 24 °C for 30 min. Following addition of cells incubations were carried out at 37 °C for 60 min in the presence of O_2/CO_2 (95/5). At the end of incubation samples were processed for cAMP determination as described in Experimental Procedure. Results are the mean of triplicate samples on duplicate determinations ± S.E.M. Bovine brain mixed gangliosides were used in this study

Additions	cAMP
	pmol · μg DNA^{-1}
None	1.7 ± 0.09
Mixed gangliosides (500 μg/ml)	1.0 ± 0.04
Cholera enterotoxin (10 ng/ml)	6.33 ± 0.33
Cholera enterotoxin (1 μg/ml)	14.92 ± 0.38
Choriogonadotropin (10 ng/ml)	8.93 ± 0.30
Choriogonadotropin (1 μg/ml)	12.52 ± 0.51
Lutropin [NIH-LH B9] (1 μg/ml)	6.69 ± 0.28
Lutropin [NIH-LH S18] (1 μg/ml)	5.74 ± 0.11
Cholera enterotoxin (10 ng/ml) + mixed gangliosides (500 μg/ml)	0.92 ± 0.05
Cholera enterotoxin (1 μg/ml) + mixed gangliosides (500 μg/ml)	1.13 ± 0.06
Choriogonadotropin (10 ng/ml) + mixed gangliosides (500 μg/ml)	8.10 ± 0.43
Choriogonadotropin (1 μg/ml) + mixed gangliosides (500 μg/ml)	11.32 ± 0.2
Lutropin [NIH-LH-B9] (1 μg/ml) + mixed gangliosides (500 μg/ml)	5.73 ± 0.23
Lutropin [NIH-LH-S18] (1 μg/ml) +mixed gangliosides (500 μg/ml)	5.81 ± 0.44

Table 4. *Lack of effect of purified gangliosides and mixed gangliosides of various composition on ^{125}I-labelled choriogonadotropin binding to rat ovarian cells*

Incubation conditions were the same as described in Fig. 1. Gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1} were isolated from brain mixed ganglioside preparation (ICN Pharmaceuticals) by preparative thin-layer chromatography [61]. Gangliosides from rat ovary and bovine corpus luteum were isolated according to Leeden et al. [61] as described under Experimental Procedure. Rat brain gangliosides were isolated according to Suzuki [62]. The results are expressed as the mean of triplicate samples on duplicate determinations ± S.E.M.

Addition		^{125}I-labelled choriogonadotropin bound
	μg/ml	counts × min^{-1} × 100 μg DNA^{-1}
Experiment 1 (Individual gangliosides)		
None	–	7612 ± 281
G_{M1}	20	8736 ± 325
	100	8050 ± 328
G_{D1a}	20	8258 ± 219
	100	8179 ± 122
G_{D1b}	20	8187 ± 759
	100	8113 ± 342
G_{T1}	20	7888 ± 382
	100	7455 ± 176
Choriogonadotropin	0.02	3656 ± 784
Experiment 2 (Mixed gangliosides)		
None	–	8939 ± 430
Rat ovary	50	8029 ± 4
	200	8259 ± 262
Bovine corpus luteum	50	7510 ± 75
	200	8141 ± 151
Rat brain	50	7845 ± 187
	200	7961 ± 142
Bovine brain	200	8080 ± 534
Choriogonadotropin	0.01	3785 ± 62

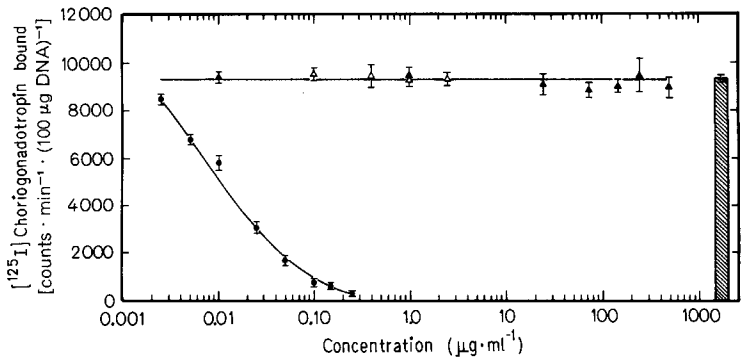

Fig. 2. *Lack of cholera enterotoxin and gangliosides effect on ^{125}I-labelled choriogonadotropin binding of rat ovarian cells.* ^{125}I-labelled choriogonadotropin (4 ng, 160000 counts/min) was preincubated with cholera enterotoxin, bovine brain mixed gangliosides or unlabelled choriogonadotropin for 30 min under the conditions described in Fig. 1. Following preincubation, 0.1 ml cells (5×10^6 cells) were added and incubation continued for 30 min, after which cells were processed for the determination of bound radioactivity. Other details have been described in Experimental Procedure. (△——△) Cholera enterotoxin; (▲——▲) gangliosides; (●——●) unlabelled choriogonadotropin. Hatched bar, ^{125}I-labelled choriogonadotropin binding without any test substance. Results are mean of triplicate samples on duplicate determinations ± S.E.M.

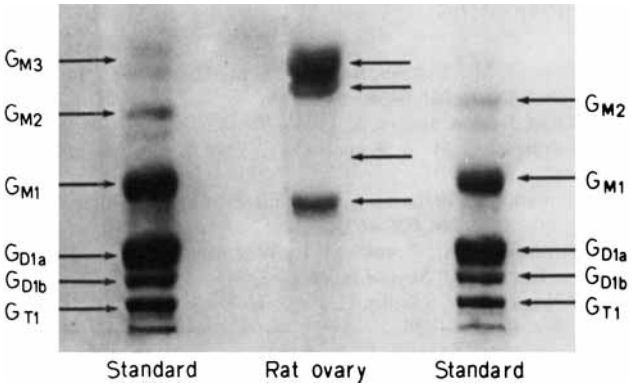

Fig. 3. *Thin-layer chromatography of ganglioside extract of ovarian cells.* Ganglioside extraction, purification and thin-layer chromatographic separation were carried out as described in Experimental Procedure

G_{M3}. A significant amount of resorcinol-positive material from ovarian cell extract also migrated between gangliosides G_{M2} and G_{M3}, and other faint resorcinol-positive material co-migrated with authentic ganglioside G_{M1}. Some resorcinol-positive material migrated between gangliosides G_{D1a} and G_{M1}.

Pretreatment of Ovarian Cells with Gangliosides on Subsequent Steroidogenesis in Response to Lutropin, Choriogonadotropin and Cholera Enterotoxin

Since exogenous gangliosides can be spontaneously incorporated into the cell membrane [43,48,70], we tested the pretreatments of ovarian cells with gangliosides on subsequent steroidogenesis stimulated by gonadotropins and cholera enterotoxin. Pretreatment of ovarian cells with gangliosides did not result in any significant alterations of subsequent steroidogenic response elicited by any stimulator. Even very low concentrations of choleragen (2.5 ng/ml) failed to show enhanced steroidogenesis in cells pretreated with gangliosides. (Data not shown.)

DISCUSSION

An important step in the regulation of ovarian function by gonadotropin is the initial interaction of hormone with the receptor and the modulation of this process by other membrane constituents present in the intact cell. The use of cholera enterotoxin as a membrane probe has added new directions in the area of hormone-receptor interaction in the cell membrane and subsequent biological response. The initial step in the action of the toxin is the binding of the β subunit to the membrane receptor which is presumably the monosialoganglioside, G_{M1} [32–34,36–49], with the resultant stimulations of adenylate cyclase and cAMP in a wide variety of cell types [37,49,50, 68–75]. In the present studies we have explored whether gangliosides or ganglioside-like structure are the part of membrane receptors for gonadotropin in ovary. We also examined the effect of gonadotropin-ganglioside interaction on the subsequent biological responses by hormone.

Since gangliosides are normal cell membrane constituents whose carbohydrate moieties are exposed to the outside of the cells, it is likely that the previously observed gonadotropin-ganglioside interaction [56–58] may involve the binding to the carbohydrate portion of the gangliosides on the cell membrane. The failure of gangliosides to inhibit ^{125}I-labelled choriogonadotropin binding to ovarian cells suggests that the tropic hormone receptors in the present system are very specific and that the hormone receptor-interaction is a more complex phenomenon. These findings coupled with results on the lack of ganglioside effect on cAMP and progesterone responses suggest that the tropic hormone exerts its effect through a different or additional mechanism from that of cholera enterotoxin, the nature of which remains to be determined. Recently, ganglioside inhibition of gonadotropins binding to rat testis plasma membrane has been described, in which the inhibition was shown to be the result of interaction between the hormone and the gangliosides rather than the membrane and glycolipids [57,58]. However, it was not clear whether the inhibition in gonadotropin binding to rat testis plasma membranes also parallels a corresponding change in the cell response. Since gangliosides G_{D1a}, G_{D1b}, G_{T1} and G_{M1} interact with choriogonadotropin and lutropin to a greater extent than the other gangliosides, the amount and type of bovine brain ganglioside [28, 47, 57, 58, 62, 63, 65] used as well as other gangliosides in the present experiments suggest that the ineffectiveness of these agents on gonadotropin-stimulated responses cannot be attributed to the nonavailability of the ganglioside to produce an effect. The inability of various purified gangliosides (Table 5) as well as gangliosides of different composition (Table 6) to modulate ^{125}I-labelled choriogonadotropin binding activity further support the lack of involvement of gangliosides in gonadotropin receptor interaction. The inhibition of cholera enterotoxin stimulated cAMP and progesterone responses by exogenous gangliosides suggests that these agents were indeed effective. The observations further signify the complex nature of hormone-receptor interaction and subsequent biological responses. The present study of ganglioside inhibition of cholera enterotoxin stimulated biological responses in ovarian cells agrees well with those reported for inhibition of biological effects of toxin

by gangliosides in other biological systems [38–47, 50].

The lack of cholera enterotoxin effect on ^{125}I-labelled choriogonadotropin binding to ovarian cells suggests that the binding sites of choleragen and gonadotropins are different. Since maximum stimulatory doses of toxin did not modify the maximum progesterone production caused by choriogonadotropin (unpublished results), the toxin effect may be brought about by the secondary changes within the cells, possibly through an increase of cAMP. Furthermore, prior treatment of cells with gangliosides did not increase subsequent steroidogenesis promoted by either lutropin, choriogonadotropin or cholera enterotoxin. Previously Cuatrecasas [49] has demonstrated that the lypolytic action of cholera enterotoxin can be enhanced by prior treatment of fat cells with gangliosides. More recently Moss et al. [72] reported that G_{M1}-deficient mouse fibroblasts that were unresponsive to choleragen when grown in chemically defined medium became responsive after binding exogenous ganglioside G_{M1}. However, binding of comparable amounts of gangliosides G_{M3}, G_{M2} and G_{D1a} to the cells did not restore responsiveness. Although these studies strongly support ganglioside G_{M1} as the possible receptor for choleragen on cell surface receptors, there are reports which do not totally support this hypothesis. Kanfer et al. [48] recently carried out an extensive study on choleragen regulation of lypolysis in fat cells and their findings could not support ganglioside G_{M1} as a native receptor for choleragen on these cells. Similarly, King et al. [76] attempted to correlate the quantitative relationship between the amount of toxin bound to pigeon erythrocyte membrane and the extent of gangliosides G_{M1} stimulation of cholera enterotoxin activation of adenylate cyclase and suggested that the natural receptor for cholera enterotoxin may be more complex than ganglioside G_{M1}. Donta et al. [77] also investigated the role of gangliosides in relation to cholera enterotoxin stimulated steroidogenesis in adrenal tumor cells and concluded that ganglioside G_{M1} may not be a true representative receptor for choleragen in his system.

From the various findings reported in the present communication, it is concluded that gonadotropin binding and the subsequent physiological responses were not influenced by gangliosides directly. Furthermore, gonadotropin receptor interactions and subsequent biological responses in ovarian cells probably represent a more complex instance in which cells specially respond to receptor-mediated gonadotropin signals.

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