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RECEPTOR-MEDIATED GONADOTROPIN ACTION IN THE OVARY

DEMONSTRATION OF ACUTE DEPENDENCE OF RAT LUTEAL CELLS ON EXOGENOUSLY SUPPLIED STEROID PRECURSOR (STEROLS) FOR GONADOTROPIN-INDUCED STEROIDOGENESIS

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Incubation of luteal cells with human, horse and rat sera, but not bovine sera resulted in enhanced basal and hCG-stimulated progesterone accumulation. The stimulatory effect of human or rat sera on basal, hCG- or 8 Br-cyclic AMP-induced progesterone synthesis in luteal cells was evident within 15–30 min after incubation, reaching a maximum after 3–4 h. The stimulatory effects of hCG and/or sera were blocked by inhibitors of RNA and protein synthesis. Similarly, lysosomotropic agents, chloroquine (100 μM) and ammonium chloride (10 mM), partly blocked the steroidogenic response of luteal cells to hCG and/or human or rat sera. Incubation of cells in the presence of 2-deoxyglucose, sodium azide and phenylmethylsulfonyl fluoride resulted in partial inhibition of progesterone secretion in response to hCG or sera. Fractionation of human or rat sera into various lipoprotein fractions demonstrated that LDL and HDL most effectively supported and potentiated the steroidogenic response to hCG. Lipoprotein-deficient serum, however, did not alter gonadotropin-induced steroid production. Incubation of luteal cells with increasing concentrations of h-LDL and h-HDL enhanced both basal and hCG-mediated steroidogenesis in a dose-related manner, although very high concentrations of these lipoproteins were inhibitory. Further, [^{3}H]cholesterol from [^{3}H]cholesteryl linoleate-LDL was incorporated into luteal cell progesterone and the extent of this incorporation was enhanced by hCG. Addition of excess unlabeled h-LDL, h-HDL, as well as r-HDL, drastically reduced the incorporation of radioactive label into progesterone. These studies suggest that (a) serum potentiation of steroidogenesis was due to presence of lipoporteins, mainly LDL and HDL, and (b) the lipoprotein-bound cholesterol is delivered into the luteal cells and utilized for steroidogenesis.

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Abbreviations: 8 Br-cyclic AMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; hCG, human chorionic gonadotropin (choriogonadotropin); h-VLDL, human very low density lipoprotein; h-LDL, human low density lipoprotein; h-HDL, human high density lipoprotein; LPDS, lipoprotein-deficient serum; r-VLDL, rat very low density lipoprotein; r-LDL, rat low density lipoprotein; r-HDL, rat high density lipoprotein; 4-APP, 4-aminopyrazolo[3,4-d]pyrimidine; MIX, 1-methyl-3-isobutylxanthine; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(2-(5-phenyloxazolyl)benzene.

Introduction

The steroid-producing tissues, adrenals, testis and ovary, exhibit greater requirements for cholesterol compared to nonsteroid-secreting tissues. These tissues utilize cholesterol not only for membrane biogenesis but also for steroid hormone production. In ovary, cholesterol for steroidogenesis can be made available by three principle sources: (a) synthesis de novo from acetate [1–4], (b) hydrolysis of esterified cholesterol esters stored in lipid droplets [1,3,4], and (c) uptake of preformed cholesterol from blood

[3,5]. Although these sources are capable of supplying cholesterol for steroidogenesis, their relative contribution and interrelationship under normal physiological conditions is not well understood at the present time. Further, while the initial events in gonadotropin-induced steroidogenesis including hormone binding and adenylate cyclase activation have been studied extensively in recent years [4,6,7], the precise knowledge of the role of precursor in the steroidogenic process is still fragmentary.

Recently several investigators have demonstrated that lowering of blood cholesterol levels by the pharmacological agent, 4-aminopyrazolo[3,4-d]pyrimidine (4-APP), leads to a reduction in the circulating level of steroid hormones [8–11]. While these studies suggest a possible link between in vivo steroidogenesis and blood cholesterol levels, the 4-APP manipulation of cholesterol levels may not represent a true physiological situation and, thus, this still leaves an open question about the principal source of the substrate for steroidogenesis. Preliminary studies reported from this laboratory suggest that incubation of ovarian cells in the presence of fetal calf serum not only shifted the dose response curve for hCG and LH, but also led to enhanced maximal accumulation of progesterone [12]. These observations prompted us to: (1) Determine the stimulatory effects of sera from different sources for steroidogenesis, (2) identify factors from serum which cause stimulation of steroidogenesis and (3) study the extent of utilization and regulatory role of endogenous versus exogenous cholesterol in gonadotropin-induced steroidogenesis. Rat luteal cells prepared from highly luteinized ovaries [13,14] were used in the present studies. These cells are highly sensitive to physiological doses of gonadotropins and respond to LH/hCG with enhanced accumulation of cyclic AMP and progesterone.

We report here that in luteal cells (a) sera from different sources stimulated steroidogenesis in a time- and concentration-dependent manner, (b) the major stimulatory factors in serum were identified as LDL and HDL, (c) cholesterol from radioactively labeled h-LDL was incorporated into progesterone and (d) these cells are acutely dependent upon exogenous cholesterol source in the form of lipoproteins for maximum expression of steroidogenic response.

Materials and Methods

Materials

Purified human choriogonadotropin (CR-119, 11 500 I.U./mg) was generously supplied by Dr. R. Canfield, Columbia University, New York, through Population Research Branch, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD. 8-Br-cyclic AMP, cyclic AMP and cyclic AMP-dependent protein kinase (beef heart) were obtained from Sigma Chemical Co., St. Louis, MO. 1-Methyl-3-isobutylxanthine (MIX) and cyclic [8-^{3}H]AMP (spec. act. 25 Ci/mmol) were purchased from Aldrich Chemical Co., Milwaukee, WI, and Amersham/Searle, respectively. Collagenase (Type CLS) and deoxyribonuclease were supplied by Worthington Biochemical Corporation, Freehold, NJ. Medium 109/Medium 199, fetal bovine and horse sera were products of Grand Island Biological Co., Grand Island, NY. Other reagents used were of analytical reagent grade.

Methods

Hormonal pretreatment of rats. 22–24-day-old female rats, obtained from Spartan Research Animals, Inc., Hazlett, MI, were used in the present studies. Highly luteinized ovaries from these rats were obtained following the regimen described by Parlow [15]. This involved subcutaneous injection with 50 I.U. pregnant mare's serum gonadotropin (Gesty, Organon, Oss, Holland) followed 56 h later by 25 I.U. of hCG (Sigma Chemical Co). on day 0.

Preparation of luteal cells. 5–7 days after the injection of hCG, the animals were killed by cervical dislocation, the ovaries removed, freed of connective tissues, weighed and placed in Medium 109 containing 0.1% bovine serum albumin. Collagenase-dispersed luteal cells were prepared by a slight modification of the procedure described earlier by workers from this laboratory [13,14]. Briefly, the ovaries were cut into small pieces (approximately 1 mm^3) with scissors and transferred to a 50 ml plastic beaker. The tissue was suspended at a concentration of 50 mg/ml of Medium 109/0.1% bovine serum albumin containing 500 U/ml collagenase and 4 mg/ml DNAase. The samples were incubated for 60 min at 37°C in an atmosphere of O_2/CO_2 (95 : 5, v/v). At 30 and 60 min of incubation the cells were dissociated by flushing the tissue and

medium 30–40 times through a 1 ml plastic syringe. At the end of the incubation the cell suspension was diluted with 2 vol. of medium and centrifuged at 600 × *g* for 3 min. The sedimented cells were washed two times in Medium 109/0.1% bovine serum albumin and then resuspended to a concentration of approximately $1 \cdot 10^7$ cells/ml. The viability of the cells ranged between 80–90% as ascertained by the trypan blue exclusion test. DNA was determined according to the procedure of Burton [16].

Preparation of ovarian cells. Cells from whole ovaries of 24–26-day-old rats were prepared essentially as described previously by workers from this laboratory [17–20]. Incubation conditions for the measurement of progesterone response were the same as described below for luteal cells.

Incubation conditions for progesterone measurement. Unless otherwise stated aliquots of luteal cells (approximately $2 \cdot 10^6$ cells in 0.1 ml) were transferred into 12 × 75 mm tubes containing 0.3 ml Medium 109/0.1% bovine serum albumin, and, where required, appropriate concentrations of various sera, hCG, or lipoproteins were also added. The incubations were carried out at 37°C in a Dubnoff metabolic shaking incubator for 4 h in the presence of O_2/CO_2 (95 : 5, v/v). Following incubation, the reaction was stopped by placing the sample tubes in a boiling water bath for 3 min. The samples were then diluted with 0.6 ml H_2O and 10000 cpm [1,2(n)-^{3}H]progesterone (spec. act 55 Ci/mol) were added to monitor progesterone recovery and the samples left in the cold overnight. Next morning the samples were extracted with light petroleum ether and assayed for progesterone by radioimmunoassay as described earlier [12,17, 19].

Measurements of cyclic AMP accumulation in luteal cells. Luteal cells (approx. $2 \cdot 10^6$) were dispersed in a final volume of 0.4 ml of Medium 109/0.1% bovine serum albumin, 0.5 mM MIX, and where required, 25 ng/ml hCG and various sera (25%, v/v). Following incubation, usually for 2 h, the reaction was stopped by placing tubes in a boiling water bath for 5 min and then transferred to ice. After 10 min, 0.5 ml trichloroacetic acid (10%, w/v) and 0.1 ml of cyclic [^{3}H]AMP (approx. 2000 cpm) were added and the samples stored overnight in the cold. After centrifugation at 3000 × *g* for 10 min, the clear supernatants were processed and assayed for cyclic AMP by the procedure of Gilman [21] as described earlier [18].

Incorporation of [^{3}H]cholesterol from [^{3}H]-cholesteryl linoleate-hLDL into progesterone by luteal cells. Rat luteal cells (approx. $2 \cdot 10^6$) were incubated in a final volume of 1 ml of Medium 109 containing 1 mg/ml bovine serum albumin, 20 μg protein/ml [^{3}H]cholesteryl linoleate-hLDL (60000 cpm/nmol), cholesteryl linoleate and, where required, 10 ng/ml hCG, 720 μg protein/ml h-LDH, 750 μg/protein/ml h-HDL or 350 μg protein/ml r-HDL. After incubation for 4 h in an atmosphere of O_2/CO_2 (95 : 5), 1 ml phosphate-buffered saline was added to each tube and the tubes were transferred to boiling water bath for 3 min. [^{14}C]Progesterone (50 μg, 1000 cpm), 20α-hydroxypregn-4-en-3-one (50 μg) and pregnenolone (50 μg) were added as carriers and steroids were extracted two times with 5 vol. of light petroleum ether and two times with 5 vol. of ethyl acetate. The petroleum ether and ethyl acetate fractions were dried separately under N_2. The residues from two fractions were dissolved in chloroform/methanol (2 : 1), combined and redried under a stream of N_2. The residues were dissolved in 3 ml of 90% aqueous methanol and partitioned three times against 1 ml of hexane [10,22]. Steroids in the methanolic phase were separated by thin-layer chromatography using Silica Gel G glass plates. The plates were developed either one time in a solvent system of chloroform/diethyl ether (9 : 1, v/v), [21], three successive times in a system of isopropylether/diethyl ether/acetic acid (70 : 30 : 2, v/v) [10] or three times in a solvent system of isopropyl ether/petroleum ether/acetic acid (70 : 20 : 1, v/v). Authentic standards, cholesteryl oleate, cholesterol, progesterone, 20α-hydroxypregn-4-en-3-one and pregn-5-en-3-ol-20-one, were always run simultaneously and steroids were visualized by exposure to iodine vapor. The steroids were eluted in chloroform/methanol (2 : 1) and counted for radioactivity determinations. In some cases separation of progesterone, pregnenolone, and 20α-hydroxypregn-4-en-3-one was achieved by thin-layer chromatography on plastic-backed Silica Gel G sheets using chloroform/diethyl ether (9 : 1) as a developing solvent system [23]. The spots were cut out and counted for radioactivity after the addition of PPO/POPOP-based scintillation fluid.

Isolation of lipoproteins. Human VLDL ($d <$

1.006 g/ml) LDL (d = 1.02–10.5 g/ml), HDL (d = 0.07–1.215 g/ml) and lipoprotein-deficient serum ($d > 1.215$ g/ml) were prepared by differential ultracentrifugation, using KBr for density adjustments [24]. Human lipoprotein fractions were isolated from blood plasma from healthy donors and collected in 0.1% EDTA. Rat lipoprotein fractions were isolated from pooled sera. Isolated fractions were concentrated by recentrifugation at their respective densities at 45 000 rpm in a Ti 60 rotor for 24 h at 11°C. Lipoproteins were dialyzed for 36–48 h against three to four changes of 0.15 M NaCl containing 0.3 mM EDTA, sterilized by filtration through a 0.22 μm Millipore filter and stored at 4°C until used. Immediately prior to use, these fractions were dialyzed against 0.15 M NaCl to remove EDTA. Purity of lipoprotein fractions was checked by cellulose acetate [25] and agarose gel electrophoresis in 0.05 M barbital buffer, pH 6.8, using precast (Bio-Gram A, BioRad Lab) agarose slides [26]. The mass ratio of total cholesterol to protein was 1.4 : 1 and 1 : 3.58 for human LDL and human HDL, respectively. Similarly, rat LDL and rat HDL had mass ratios of total cholesterol to protein of 1.3 : 1 and 1 : 1.6, respectively. Human LDL radioactively labeled with [^{3}H]cholesteryl linoleate was prepared according to the procedure of Kreiger et al. [27]. Total plasma or serum cholesterol was determined by the procedure of Zak [28]. For lipid analysis, lipids were extracted from lipoprotein fractions in chloroform/methanol (2 : 1, v/v) [29]. Lipoprotein phospholipid phosphorous was determined by the procedure described previously [30]. The relative contribution of individual phospholipids to total phospholipids and free cholesterol and cholester ester to total cholesterol was determined after the separation of individual lipids by thin-layer chromatography [31]. The separation of neutral lipids including cholesterol and cholesterol esters was accomplished by single-dimension, two-step development thin-layer chromatography [31] using isopropyl ether/acetic acid (96 : 4, v/v) as first developing solvent and petroleum ether/diethyl ether/acetic acid (90 : 10 : 1, v/v) or diethyl ether/hexane (6 : 94, v/v) as second solvent. Cholesterol and cholesterol esters were eluted successively with diethyl ether (20 ml), chloroform/methanol (10 ml, 4 : 1, v/v) followed by chloroform/methanol (10 ml, 2 : 1, v/v). Cholesterol esters, after saponification in alcoholic KOH, and free cholesterol were quantitated by the colorimetric procedure of Zak [28] and, if necessary, by the micro method of Glick et al. [32]. Protein content of lipoprotein was determined by a modification of the procedure of Lowry et al. [33] as described by Markwell et al. [34].

Results

Effect of different incubation media. Results presented in Table I demonstrate the effect of various incubation media and human sera on progesterone production in luteal cells in response to hCG. Exposure of luteal cells with hCG in Medium 109 (Eagles minimal essential medium with Earles salt) caused a 6–7-fold increase in progesterone production. Similarly, gonadotropin incubation in Medium 199 also resulted in a 6–7-fold stimulation of progesterone accumulation. Since Medium 199 also contains 200 ng/ml cholesterol it is clear that direct addition of steroid precursor in incubation medium had no effect on steroidogenesis. In contrast, direct addition of human (male) serum enhanced steroidogenesis almost 2-fold over the values obtained without added serum and in the presence of hCG. The small difference between basal and hCG-stimulated steroid production in the presence of serum suggests that human serum contains endogenous LH type of activity, which is sufficient to stimulate steroidogenesis maximally. In some studies exogenously added hCG did not show any stimulatory effect on human serum-stimulated steroidogenesis, presumably due to very high endogenous levels of LH. By contrast, rat sera from 26-day-old rats which contains comparatively lower amount of LH exhibited marked difference between basal and hCG-stimulated progesterone production (Table I).

Effect of increasing concentrations of various sera. The results presented in Fig. 1 demonstrate the effect of increasing concentrations of human, horse and fetal bovine serum on luteal cell steroidogenesis. Addition of human or horse serum enhanced progesterone accumulation in a concentration-dependent manner. Concentrations as low as 2.5% significantly enhanced steroid synthesis. Further increase in serum concentration resulted in enhanced steroidogenesis, the maximal effective serum concentration being 25–40%. At maximum effective concentrations, these two sera caused a net 2-fold increase in progesterone

TABLE I

INFLUENCE OF THE COMPOSITION OF THE INCUBATION MEDIA ON GONADOTROPIN-STIMULATED STEROID SYNTHESIS IN RAT LUTEAL CELLS

Rat luteal cells from pseudopregnant rat ovaries were prepared in Medium 109/0.1% bovine serum albumin as described in Materials and Methods. For experiment 1, aliquots of cells (approx. $2 \cdot 10^7$) in triplicate were suspended and washed three times by centrifugation either in Medium 109/0.1% bovine serum albumin or Medium 199/0.1% bovine serum albumin and finally suspended in respective media at a concentration of $2 \cdot 10^7$ cell/ml. Aliquots of cells (0.1 ml) were incubated without or with hCG in a final volume of 0.4 ml of the media for 4 h in the presence of O_2/CO_2 (95: 5, v/v) at 34°C. For experiment 2, the cells were incubated with various sera in the presence and absence of hCG for 4 h at 34°C in the presence of O_2/CO_2 (95: 5, v/v). Other details were similar to those described in Materials and Methods. The results are mean ± S.E. of three separate experiments on triplicate determinations.

Additions	Progesterone (ng/μg DNA)	
	Basal	hCG (10 ng/ml)
A. Experiment 1		
Luteal cells incubated in Medium 109/0.1% bovine serum albumin	0.54 ± 0.02	3.88 ± 0.10 ($P < 0.005$)
Luteal cells incubated in Medium 199/0.1% bovine serum albumin	0.63 ± 0.02	3.61 ± 0.05 ($P < 0.001$)
B. Experiment 2 (Incubations in Medium 109/0.1% bovine serum albumin)		
None	0.66 ± 0.09	3.24 ± 0.07 ($P < 0.005$)
Human serum (15%, v/v)	6.09 ± 0.39	7.18 ± 0.19 ($P < 0.05$)
Rat serum (15%, v/v)	1.74 ± 0.02	6.94 ± 0.62 ($P < 0.001$)
Fetal bovine serum (15%, v/v)	1.16 ± 0.02	4.64 ± 0.52 ($P < 0.001$)

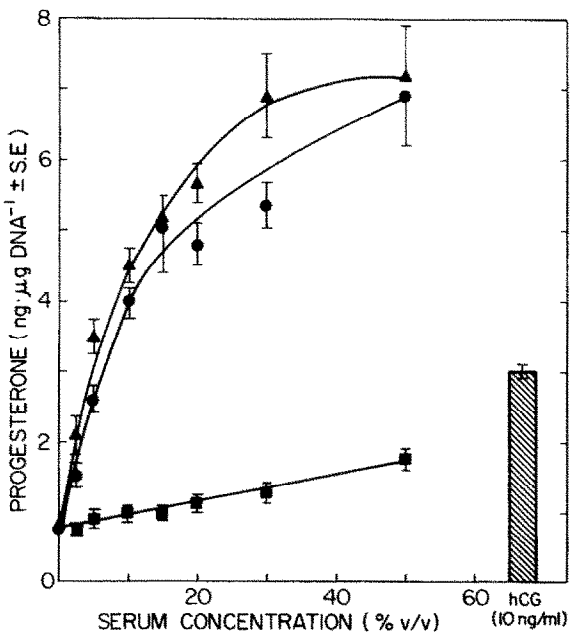

Fig. 1. Effect of increasing concentrations of human, horse and fetal bovine sera on progesterone synthesis in rat luteal cells. Aliquots of cells (approx. $2 \cdot 10^6$), in triplicate, were incubated with indicated (final) concentrations of human (●), horse (▲) or fetal bovine (■) sera. Where required, hCG (10 ng/ml) was also added. After incubation at 37°C in an atmosphere of O_2/CO_2 (95 : 5, v/v) for 4 h, the samples were extracted with light petroleum ether and quantitated by radioimmunoassay. The results are expressed as ng progesterone. μg DNA^{-1} ± S.E. and corrected for the amount of progesterone, if any, present initially in the added sera. The results are mean of three separate experiments on duplicate determinations.

accumulation compared to values obtained with hCG alone. In contrast, fetal bovine serum was not very effective and caused a slight increase in progesterone production at a concentration of 30–50%.

Effect of incubation time on sera, hCG- and 8 Br-cyclic AMP-stimulated steroidogenesis in rat luteal and rat ovarian cells. The results presented in Fig. 2 show the effect of increasing incubation time on luteal cell steroidogenesis in response to different stimuli. The basal production of progesterone rapidly increased after 30 min of incubation and ten increased slightly up to 3 h of incubation. Addition of hCG (10 ng/ml) alone showed a lag period of 10–15 min and the extent of stimulation of steroidogenesis was enhanced in a time-dependent manner, reaching to maximum after 2–3 h of incubation. Inclusion

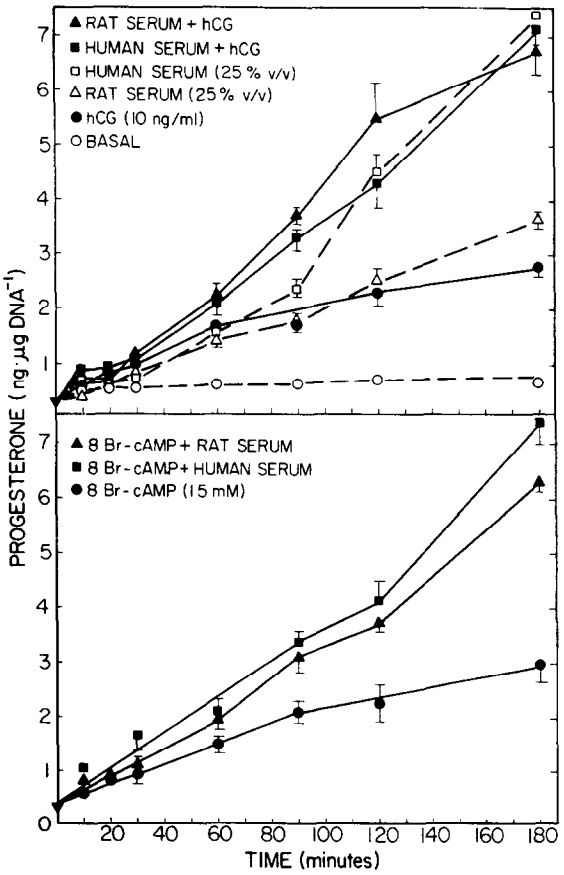

Fig. 2. Effect of increasing incubation time on human serum-, rat serum-, hCG- and 8 Br-cyclic AMP-stimulated progesterone accumulation in luteal cells. Aliquots of luteal cells (approx. $2 \cdot 10^6$) were incubated with or without hCG (10 ng/ml) 8 Br-cyclic AMP (1.5 mM) either in the absence or in the presence of rat or human sera (25%, v/v). Following incubation at 34°C for the indicated time points in an atmosphere of O_2/CO_2 (95 : 5, v/v), the samples were processed for progesterone measurements by radioimmunoassay. Other details were the same as described in Materials and Methods. The results are mean of three separate experiments on duplicate determinations.

of 25% rat serum in the incubation medium greatly potentiated the accumulation of progesterone in luteal cells (Fig. 2). The stimulatory effect was evident as early as 15–30 min after the addition of serum, both in the presence of absence of hCG, and continued to increase with increases in incubation time up to 180 min of incubation. The rat serum, however, maximally stimulated steroidogenesis after 4 h of incubation (data not shown). Luteal cell incubation with human serum alone stimulated steroidogenesis in a biphasic manner. Initially, progesterone accumulation increased linearly from 30 to 90 min and then synthesis abruptly increased at 2 h, and continued to rise up to 3 h. In contrast, addition of hCG along with human serum resulted in linear increase of steroid synthesis after 30 min up to 3 h of incubation. However, the stimulatory effect of hCG, at least in this instance, was observed only up to 90 min. Further experiments were performed to ascertain if 8 Br-cyclic AMP-stimulated progesterone synthesis was potentiated by rat serum, to determine the locus of action of sera on the steroidogenic process. Exposure of luteal cells to 8 Br-cyclic AMP (1.5 mM) caused a time-related increase in steroid synthesis and the extent of stimulation was comparable to that of hCG. Further addition of rat serum potentiated the 8 Br-cyclic action. These studies also suggest that serum stimulated steroidogenesis at a point distal to hormone-receptor interaction and cyclic AMP formation.

Results presented in Fig. 3 demonstrate the action of human and rat sera on gonadotropin-stimulated progesterone accumulation in ovarian cells prepared from 26-day-old rats. Addition of human or rat sera greatly enhanced hCG-stimulated progesterone production. The stimulation of steroidogenesis by serum was seen from 30 to 240 min of incubation. These studies demonstrate that ovarian cells have the capability to respond to sera from different sources, with increased accumulation of steroid in response to hCG or LH. Further experiments were then carried out to explore the mechanism by which serum factor(s) stimulate steroidogenesis. To accomplish these goals, luteal cells were utilized. The luteal cells were preferred, mainly due to the fact that (a) they are very sensitive to low physiological doses of LH/hCG, (b) they contain principally one cell type and (c) they secrete higher amounts of progesterone.

Effect of inhibitors of RNA and protein synthesis. The inhibitors actinomycin D, cordycepin, cycloheximide and emetine were tested for their ability to inhibit steroidogenesis under basal conditions and in the presence of hCG, rat serum and rat serum plus hCG. The results of these experiments are presented in Table II. In general, these compounds inhibited steroidogenesis to the same extent regardless of the

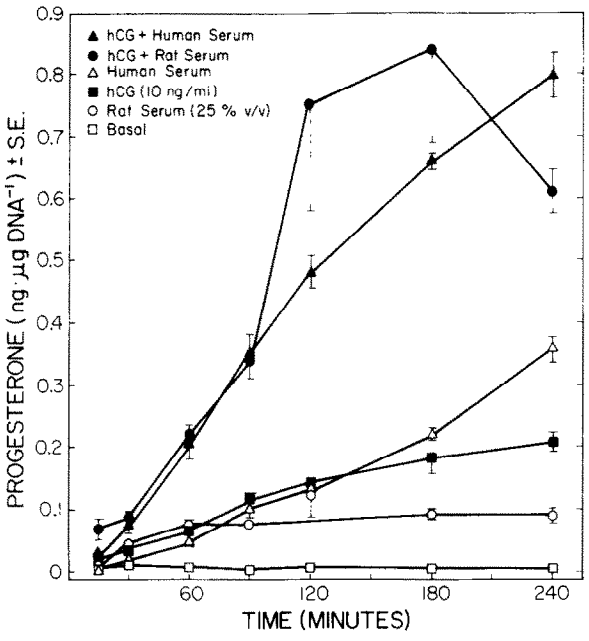

Fig. 3. Time-dependent effects of human serum, rat serum and hCG on progesterone synthesis in ovarian cells isolated from 26-day-old rats. Aliquots of ovarian cells (approx. $2 \cdot 10^6$) were incubated with and without hCG (10 ng/ml) and/or rat serum. Other details were similar to those described in Fig. 2 and under Materials and Methods. The results are mean of four separate experiments on duplicate determinations.

stimuli used for activation (e.g. hCG, serum, hCG + serum). Among these, cordycepin (250 µM) was least effective when added 30 min after the addition of stimuli. In general, protein synthesis inhibitors were more effective in blocking hormone- or serum-induced steroidogenesis. By contrast, preincubation of luteal cells with these inhibitors completely prevented the subsequent ability of the cells to respond to sera or hormones with increased accumulation of progesterone.

The time-dependent effect of emetine under different stimulatory conditions of steroidogenesis is shown in Fig. 4. As evident, these cells were very sensitive to emetine and addition of this inhibitor at time points between 10 and 15 min blocked steroidogenesis in response to various stimuli. Thus, results presented in Table II and Fig. 4 suggest that, like basal and hCG-stimulated steroidogenesis, the serum- and serum plus hCG-stimulated steroid synthesis was also sensitive to RNA and protein synthesis inhibitors. Furthermore, the stimulatory effect of serum was due to an increase in the production of progesterone and not due to decreased metabolism.

Effect of lysosomotropic agents, metabolic inhibitors and protease inhibitors. The results presented so far suggest that stimulation of steroidogenesis by sera is mediated by an intracellular site of action, possibly by making available extra cholesterol substrate. Since

TABLE II

EFFECT OF MACROMOLECULAR SYNTHESIS INHIBITORS ON RAT SERUM-STIMULATED PROGESTERONE PRODUCTION IN LUTEAL CELLS

Incubation conditions were similar to those described in Fig. 2 except that the indicated concentrations of inhibitors were added 30 min after the addition of hCG and/or serum. Total incubation time was 4 h. The results are ± S.E. of two separate experiments on triplicate determinations.

Inhibitors	Progesterone (ng/µg DNA)			
	Basal	hCG (10 ng/ml)	Rat serum (25% v/v)	Rat serum + hCG (10 ng/ml)
None	1.12 ± 0.01	5.51 ± 1.1 [a]	4.13 ± 0.47 [b]	10.25 ± 1.68 [c]
Actinomycin D (10µM)	0.76 ± 0.08	2.17 ± 0.04	1.73 ± 0.16	5.41 ± 0.52
Cordycepin (250 µM)	1.00 ± 0.09	3.18 ± 0.15	3.85 ± 0.14	7.71 ± 0.17
Cycloheximide (50 µM)	0.70 ± 0.04	1.61 ± 0.11	1.45 ± 0.21	2.57 ± 0.06
Emetine (10 µM)	0.69 ± 0.01	1.14 ± 0.06	1.32 ± 0.08	1.68 ± 0.19

[a] $P < 0.010$. [b] $P < 0.005$. [c] $P < 0.001$.

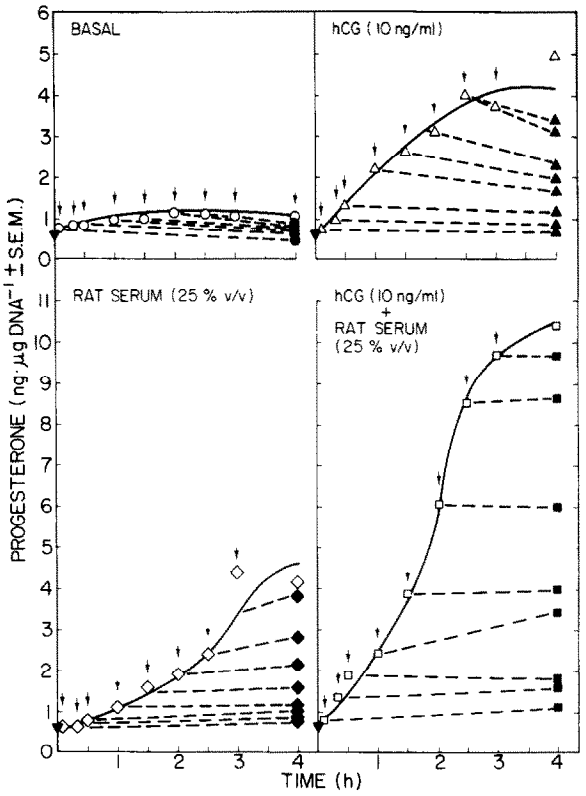

Fig. 4. Effect of addition of emetine at different time points on hCG- and rat serum-stimulated steroidogenesis in rat luteal cells. Incubation conditions were similar to that described in Fig. 2 except that at time points indicated by arrow 10 μM emetine was added and incubation continued for a total period of 4 h. The concentrations of hCG and rat serum used were 10 ng/ml and 25% (v/v), respectively. The results are mean of two separate experiments on triplicate determinations.

in many nonsteroidogenic cells [35–37,9] and human choriocarcinoma cells [38] cholesterol is delivered by LDL binding to cell surface receptors, followed by internalization and hydrolysis of cholesterol esters from the internalized lipoproteins by lysosomes, the present studies suggest that the mechanism of action of various inhibitors might be by inhibiting cellular endocytosis [39,40,36]. Exposure of luteal cells to lysosomotropic agents, chloroquine (100 μM) and ammonium chloride (10 mM) inhibited progesterone synthesis in response to rat serum and sera plus hormone (Table III). However, these inhibitors also inhibited steroidogenesis in response to hCG, and slightly decreased the basal accumulation of progesterone. Similarly, metabolic inhibitors, 2-deoxyglucose (10 mM) and sodium azide (10 mM), also affected the ability of luteal cells to respond to various stimuli. Sodium azide was most effective and completely blocked the hCG, rat serum and rat serum in the presence of hCG-stimulated steroid production (Table III). The presence of protease inhibitor, phenylmethylsulfonyl fluoride (2.5 mM), also resulted in the inhibition of steroidogenic response.

Lack of effect of various sera on gonadotropin stimulated cyclic AMP accumulation. The results of Table IV show a dose-dependent increase in cyclic AMP accumulation in response to hCG. However, unlike the steroidogenic action in luteal cell preparations, addition of sera did not affect the basal or hCG-stimulated cyclic AMP synthesis. These results provide additional support that sera stimulate steroidogenesis at a point distal to hormone receptor interaction and cyclic AMP formation.

Fractionation of human plasma and rat serum into various lipoprotein fractions. Human plasma and rat sera were fractionated into various lipoprotein fractions by differential ultracentrifugation. Results presented in Table V demonstrate the ability of VLDL, LDL, HDL and LPDS to modulate basal and hCG-stimulated progesterone accumulation. As expected, addition of human and rat serum potentiated steroid synthesis over that observed in the presence of hCG alone. Addition of h-VLDL and, to some extent, r-VLDL resulted in stimulation of basal steroid synthesis. These lipoproteins, however, were without any effect on hCG-induced progesterone accumulation. Exposure of luteal cells to h-LDL and h-HDL not only stimulated basal but also greatly potentiated the hCG-stimulated progesterone accumulation (Table V). Addition of isolated r-LDL and r-HDL also enhanced basal and hCG-induced steroidogenesis to varying extents. The human LPDS fraction which was devoid of lipoproteins had no significant effect. These results suggest that the stimulatory factors present in serum constitute LDL and HDL and that the luteal cells have unique ability to utilize lipoprotein-delivered cholesterol for steroidogenesis. Further, unlike in other systems [10,37,9,41], these cells have the ability to utilize lipoproteins (LDL and HDL) for steroidogenesis immediately after their isolation, with no apparent need for prolonged incu-

TABLE III

EFFECT OF LYSOSOMOTROPIC AGENTS, METABOLIC INHIBITORS AND PROTEASE INHIBITOR ON hCG- AND SERA-STIMULATED PROGESTERONE PRODUCTION IN RAT LUTEAL CELLS

Rat luteal cells (approx. $2 \cdot 10^6$) were incubated with rat serum, human serum or hCG either in the presence or in the absence of indicated concentration of various inhibitors for 4 h. Other details are similar to those described in Fig. 2. The results are mean ± S.E. of four separate experiments on duplicate determinations.

Inhibitor	Progesterone (ng/μg DNA)					
	Basal	hCG (10 ng/ml)	Rat serum (25%)	Human serum (25%)	Rat serum + hCG	Human serum + hCG
None	0.81 ± 0.02	3.48 ± 0.06	2.64 ± 0.03	6.10 ± 0.12	5.62 ± 0.04	5.64 ± 0.07
Chloroquine (100 μM)	0.76 ± 0.01	2.46 ± 0.08	1.8 ± 0.09	4.72 ± 0.12	3.96 ± 0.05	4.10 ± 0.06
Ammonium chloride (10 mM)	0.79 ± 0.04	2.88 ± 0.04	2.30 ± 0.02	4.22 ± 0.08	4.36 ± 0.05	4.0 ± 0.03
2-Deoxyglucose (10 mM)	0.78 ± 0.01	2.96 ± 0.05	1.6 ± 0.06	3.54 ± 0.12	3.68 ± 0.09	3.88 ± 0.17
Sodium azide (10 mM)	0.41 ± 0.03	0.40 ± 0.01	0.71 ± 0.01	0.90 ± 0.06	0.88 ± 0.03	0.82 ± 0.08
Phenylmethylsulfonyl fluoride (2.5 mM)	0.74 ± 0.05	0.91 ± 0.02	1.32 ± 0.06	1.82 ± 0.13	2.76 ± 0.09	2.60 ± 0.13

TABLE IV

LACK OF EFFECT OF VARIOUS SERA ON CYCLIC AMP PRODUCTION IN RAT LUTEAL CELLS IN RESPONSE TO hCG

Rat luteal cells (approx. $2 \cdot 10^6$) were exposed to the indicated concentrations of hCG, human, horse or rat sera in 0.4 ml Medium 109 containing 1 mg/ml bovine serum albumin and 0.5 mM MIX. After incubation at 34°C for 2 h in the presence of O_2/CO_2 (95 : 5, v/v) the samples were processed and assayed for cyclic AMP as described in Materials and Methods. The results are mean ± S.E. of three separate experiments on duplicate determinations. n.s., not significant.

Additions	Cyclic AMP (pmol/μg DNA)
Basal	2.08 ± 0.21
Human serum	2.27 ± 0.27 (n.s.)
Horse serum	2.07 ± 0.15 (n.s.)
Fetal bovine serum	2.16 ± 0.19 (n.s.)
Rat serum	2.04 ± 0.09 (n.s.)
hGC (0.1 ng/ml)	2.45 ± 0.10 ($P < 0.05$)
hCG (1 ng/ml)	5.50 ± 0.46 ($P < 0.005$)
hCG (10 ng/ml)	7.62 ± 0.54 ($P < 0.001$)
hCG (25 ng/ml)	8.47 ± 0.58 ($P < 0.001$)
Human serum + hCG (0.1 ng/ml)	3.16 ± 0.17 ($P < 0.01$)
Human serum + hCG (1 ng/ml)	4.52 ± 0.28 ($P < 0.005$)
Human serum + hCG (10 ng/ml)	7.83 ± 0.19 ($P < 0.001$)
Horse serum + hCG (10 ng/ml)	7.41 ± 0.45 ($P < 0.005$)
Fetal bovine serum + hCG (10 ng/ml)	7.80 ± 0.44 ($P < 0.005$)
Rat serum + hCG (10 ng/ml)	6.98 ± 0.29 ($P < 0.001$)

bation in lipoprotein-free medium.

Effect of increasing concentrations of h-LDL and h-HDL. The effect of exposure of luteal cells to increasing concentrations of h-LDL and h-HDL on steroidogenesis is shown in Fig. 5 (A, B). Addition of h-LDL from 25 μg protein/ml up to 400 μg protein/ml enhanced basal and hCG-stimulated progesterone accumulation in an almost linear manner. Higher concentrations of h-LDL, from 1 600 μg protein/ml up to 2 800 μg protein/ml, were inhibitory (Fig. 5A). Results presented in Fig. 6B demonstrate the dose-dependent effect of h-HDL on steroidogenesis. Basal steroidogenesis was maximally stimulated in the presence of 100–200 μg protein/ml of h-HDL and then remained constant up to 2 800 μg protein/ml. In contrast, h-HDL potentiated hCG-induced steroidogenesis maximally at around 800 μg protein/ml. As in the case of LDL, higher concentrations (1 600–2 800 μg protein/ml) led to inhibition of steroid accumulation (Fig. 5B).

Actions of h-LDL and h-HDL on luteal cell steroidogenesis in response to increasing concentrations of gonadotropin. Results shown in Fig. 6 demonstrate luteal cell sensitivity to increasing concentrations of hCG. Concentrations of hCG as low as 0.01 ng/ml significantly stimulated progesterone synthesis and this effect was increased with increases in gonadotropin concentration, reaching a maximum between

TABLE V

EFFECT OF VARIOUS LIPOPROTEIN FRACTIONS, HUMAN AND RAT SERA ON GONADOTROPIN-STIMULATED PROGESTERONE ACCUMULATION IN LUTEAL CELLS

Incubation conditions were identical to those described in Fig. 2 except that the indicated concentrations (μg cholesterol/ml) of various lipoprotein fractions, various sera or hCG (10 ng/ml) were also added. Other details were similar to those described in Materials and Methods. The results are mean ± S.E. of four separate experiments on triplicate determinations. n.s., not significant.

Additions	Progesterone (ng/μg DNA)		P
	Basal	hCG(10 ng/ml)	
None	0.81 ± 0.03	4.4 ± 0.18	<0.001
h-VLDL (50 μg/ml)	2.12 ± 0.12	4.25 ± 0.15	<0.010
h-LDL (930 μg/ml)	1.82 ± 0.08	10.35 ± 0.35	<0.001
h-HDL (210 μg/ml)	1.44 ± 0.09	7.65 ± 0.24	<0.005
r-VLDL (60 μg/ml)	1.06 ± 0.04	4.45 ± 0.21	<0.005
r-LDL (750 μg/ml)	1.18 ± 0.06	5.65 ± 0.09	<0.02
r-HDL (600 μg/ml)	1.53 ± 0.03	7.06 ± 0.11	<0.001
h-LDPS (25%, v/v)	4.47 ± 0.07	4.65 ± 0.19	n.s.
Human (male) serum (25%, v/v)	7.41 ± 0.29	8.57 ± 0.45	<0.05
Rat serum (25%, v/v)	2.24 ± 0.38	8.51 ± 0.41	<0.005

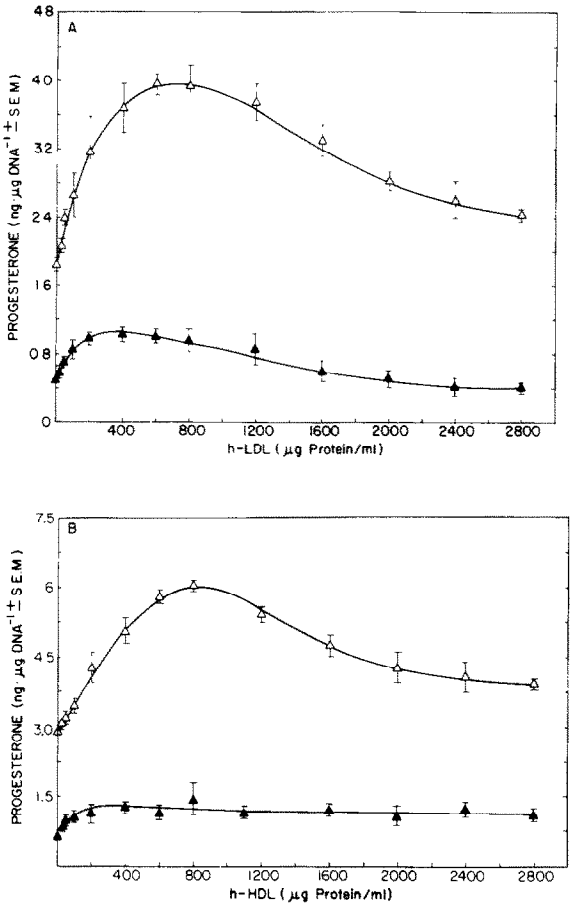

Fig. 5. Effect of increasing concentrations of human LDL and HDL on choriogonadotropin-stimulated progesterone accumulation. Rat luteal cells (approx. $2 \cdot 10^6$) were incubated with the indicated concentrations of LDL (A) or HDL (B) in the presence (△) and absence (▲) of hCG (10 ng/ml). After incubation at 34°C for 4 h in the presence of O_2/CO_2 (95 : 5, v/v), the samples (medium + cells) were extracted with light petroleum ether and progesterone was measured by radioimmunoassay. Results are mean of three separate experiments on duplicate determinations.

0.5 and 1 ng/ml. The concentration of hCG required for half-maximal stimulation of steroidogenesis (ED_{50}) was calculated to be 0.03 ng/ml. Addition of h-LDL or h-HDL potentiated the response at all the concentrations of hCG tried. The ED_{50} of hCG in the presence of h-LDL and h-HDL were calculated to be 0.012 and 0.015 ng/ml, respectively.

Incorporation of [^{3}H]cholesterol from [^{3}H]-cholesteryl-linoleate-h-LDL into luteal progesterone. Further experiments were performed to demonstrate the utilization of lipoprotein-bound cholesterol as substrate for steroidogenesis. For these experiments, h-LDL-sterol esters were replaced with [^{3}H]cholesteryl-linoleate according to the procedure of Krieger et al. [27]. Following incubation of luteal cells with [^{3}H]cholesteryl-linoleate-h-LDL in the presence and absence of hCG, the radioactively labeled progesterone was separated by thin-layer chromatography. Results presented in Table VI demonstrate the considerable radioactivity originally present in tritiated h-LDL was incorporated into the luteal cell proges-

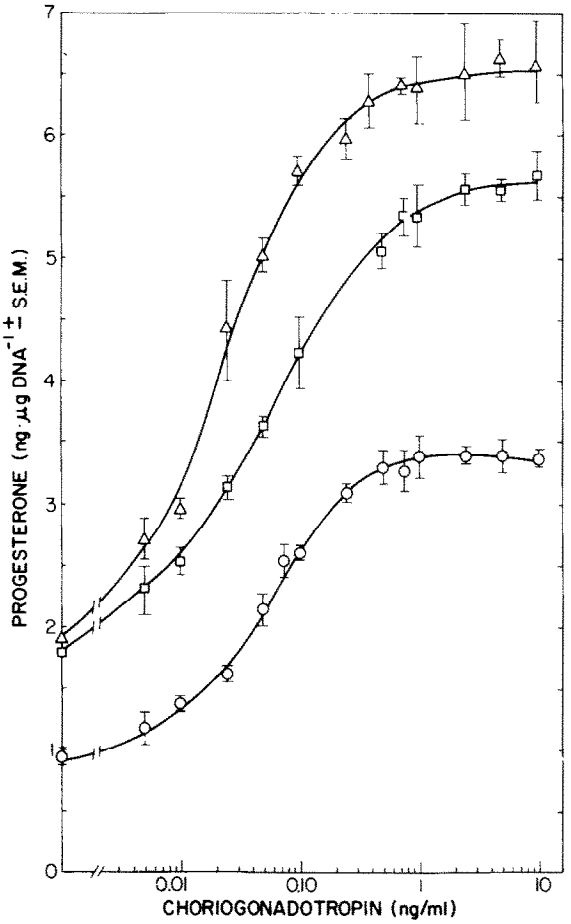

Fig. 6. Effect of human LDL and HDL on progesterone synthesis in luteal cells in response to increasing doses of hCG. Incubation conditions were identical to those described in Fig. 2 except that the indicated concentrations of hCG in the presence (□, △) and absence (○) of lipoproteins were also added. Other details were similar to those described in Materials and Methods. The results are mean of three separate experiments on duplicate determinations. □, h-HDL (800 μg protein/ml); △, h-LDL (700 μg protein/ml).

terone. The incorporation of radioactivity into 20α-hydroxy-pregn-4-en-3-one and pregnenolone compared to progesterone were 47 ± 9 and 11 ± 4%, respectively. However, since progesterone is a major steroid produced in the luteal cells in response to hCG, data dealing only with radioactivity incorporation into this product are presented. Exposure of luteal cells to ^{3}H-labeled h-LDL in the presence of hCG further enhanced 5–7-fold the incorporation of radioactivity into progesterone. Addition of excess

TABLE VI

INCORPORATION OF [^{3}H]CHOLESTEROL FROM [^{3}H]-CHOLESTERYL-LINOLEATE-hLDL INTO PROGESTERONE BY LUTEAL CELLS

Incubation conditions were similar to those described in Materials and Methods except that the indicated concentrations of unlabeled lipoproteins were also added. Thin-layer plates (Silica Gel G) were developed in a solvent system of chloroform/diethyl ether (9 : 1, v/v) and three successive developments in isopropyl ether/diethyl ether/acetic acid (70 : 30 : 2, v/v). The results are mean ± S.E. of three separate experiments. Experiments 1 and 2 were carried out with two different preparations of [^{3}H]cholesteryl-linoleate-hLDL.

Additions	[^{3}H]Progesterone (pmol radioactivity incorporated/μg DNA)	
	Experiment 1	Experiment 2
Basal	0.199 ± 0.01	0.141 ± 0.011
hCG (10 ng/ml)	0.872 ± 0.038 [a]	0.890 ± 0.018 [b]
h-LDL (720 μg protein/ml)	0.025 ± 0.007	0.017 ± 0.003
h-LDL + hCG	0.134 ± 0.010	0.146 ± 0.023
h-HDL (750 μg protein/ml)	0.018 ± 0.002	0.014 ± 0.003
h-HDL + hCG	0.145 ± 0.011	0.130 ± 0.005
r-HDL (380 μg protein/ml)	0.040 ± 0.006	0.024 ± 0.002
r-HDL + hCG	0.170 ± 0.010	0.155 ± 0.013

[a] $P < 0.005$. [b] $P < 0.001$.

unlabeled h-LDL, or r-HDL drastically reduced the extent of radioactivity incorporation into progesterone, thus suggesting that various lipoproteins could supply cholesterol to luteal cells for steroidogenesis, possibly through a receptor-mediated event(s).

Discussion

The present studies were performed to examine the mechanism involved in serum stimulation of steroidogenesis and to study the regulatory role of plasma lipoproteins in gonadotropin-induced steroidogenesis in luteal cells. Exposure of rat luteal cells to various sera enhanced the ability of these cells to produce more progesterone under basal conditions as

well as in response to gonadotropin. Although considerable species variation was observed among the sera tested, with the exception of fetal bovine serum, all other sera stimulated steroidogenesis in a time- and concentration-dependent manner. Often the stimulatory effect of hCG was not seen when cells were exposed to this hormone along with human or horse sera, although the net accumulation of progesterone was at least 2–2.5-fold higher than that seen in the presence of hCG alone. The lack of stimulatory effect of hCG in the presence of human or rat sera might be due to the fact that the steroidogenesis may be maximally stimulated by the endogenous LH in these preparations. By contrast, addition of rat serum from prepubertal rats, which contained comparatively lower amounts of LH, potentiated the stimulatory effect of hCG. Exposure of luteal cells to human, rat or fetal bovine sera neither stimulated basal production nor modified hCG-stimulated cyclic AMP accumulation. This lack of stimulation of cyclic AMP accumulation is not surprising in view of the data presented in Table IV and Fig. 6 in this communication as well as the studies reported earlier [12,14] in which the optimum concentration of trophic hormone required for maximum accumulation of cyclic AMP was much higher (10–25-fold) than that needed for maximum expression of steroidogenesis. This situation becomes clearer if one considers the effect of various sera on steroidogenesis in two different cell types (ovarian cells from immature rats vs. luteal cells) of ovarian origin as shown in Figs. 2 and 3. The whole ovarian cell suspension prepared from ovaries of 26-day-old rats responded to hCG which was further potentiated by human and rat sera. Unlike luteal cells, these cells require higher concentrations of hCG [12,14]; thus, the low endogenous LH present in the sera was not sufficient to stimulate steroidogenesis maximally. The rat serum which contained the lowest level of endogenous LH produced only a slight increase in basal steroid production, although it potentiated hCG-stimulated steroidogenesis as effectively as human serum. Further, the lack of stimulatory effect of various sera on luteal cyclic AMP production as well as their ability to potentiate 8 Br-cyclic AMP-stimulated steroidogenesis suggests that their site of action lies at a step beyond hormone receptor interaction and cyclic AMP formation.

To elucidate further the mechanism of stimulatory action of varous sera, we examined the effects of RNA and protein synthesis inhibitors, lysosomotropic agents, metabolic inhibitors and protease inhibitors on luteal cell steroidogenesis in response to hCG and/or sera. Actinomycin D, cycloheximide and emetine all blocked steroidogenic response to hCG. These results are in agreement with those reported earlier from this laboratory on gonadotropin-induced progesterone synthesis in rat ovarian cells [13], although in the present experiments these inhibitors also blocked serum-stimulated steroidogenic response. These results thus reflect a general requirement for RNA and protein synthesis that was not limited to steroidogenesis stimulated by the addition of sera. The possibility that serum stimulation of steroidogenesis involves uptake and utilization of serum cholesterol for steroid synthesis was ascertained by the use of lysosomotropic agents, metabolic and protease inhibitors. All these agents inhibited the steroidogenetic response to hCG and/or sera. Since these inhibitors partially blocked steroidogenesis in the presence and absence of serum and in response to various stimuli, it is concluded that their ability to inhibit steroidogenesis was not entirely related to their ability to modulate luteal cell cholesterol uptake from the sera, although the exact mechanism is still unknown. It has been reported that these agents inhibit Leydig tumor cell steroidogenesis in response to various stimuli [42]. Similarly, lysosomotropic agents have been shown to block steroidogenic response of ovarian cells to LH and dibutyryl cyclic AMP [43].

Using differential ultracentrifugation technique, the stimulatory factors in human and rat serum were identified mainly as HDL and LDL. In fact, exposure of luteal cells to LDL or HDL in the presence of hCG seemed to reproduce the stimulatory effect seen with sera. It is of interest to note that luteal cells appear to be unique among various steroid-producing cells [10, 37,44,38] in their ability to take up and utilize lipoprotein-delivered cholesterol for steroid synthesis without any apparent need for culture and prolonged incubation in lipoprotein-deficient medium. Thus, in contrast to cultured mouse adrenal Y-1 cells [37], cultured bovine adrenocortical cells [44], cultured rat granulosa cells [10], and cultured human choriocarcinoma cells [38], the luteal cells possess a unique capability to metabolize and incorporate lipoprotein-delivered cholesterol into progesterone, almost

immediately after their isolation from luteinized ovaries. Among various lipoproteins tested, LDL and HDL most effectively supported hCG-stimulated steroidogenesis. Further, luteal cells exhibited the ability to utilize these lipoproteins either from homologous (rat serum) or heterologous sera (human plasma) and provide a substrate for steroidogenesis. VLDL, while failing to potentiate hCG-stimulated steroid accumulation, did stimulate basal production of progesterone. Since VLDL has been shown to contain some cholesterol esters [45,46], it is quite likely that this lipoprotein could deliver a limited amount of substrate sufficient to stimulate only basal steroidogenesis.

The ability of luteal cells to utilize either LDL or HDL make them quite distinct from other steroid-secreting cultured cells [37,44,38], with the exception of cultured rat granulose cells [10]. In these systems [37,38,44], LDL was shown to be the only lipoprotein responsible for providing a substrate for steroidogenesis. In cultured adrenal [37,44] and human choriocarcinoma cells [41], specific binding of ^{125}I-labeled LDL on the cell surface was shown to be displaced in the presence of unlabeled LDL and not very effectively by HDL. While these results strongly support the possibility that the adrenal system derives cholesterol from LDL, recent reports do not entirely support this assumption [8,9]. The extensive in vivo studies reported by Anderson and Dietschy [8] suggest that the three rat steroidogenic tissues, adrenal gland, ovary and testis, preferentially take up and utilize HDL rather than LDL. In contrast, mouse adrenal glands were shown to obtain cholesterol from two lipoprotein systems, one specific for LDL and the other for HDL [9]. The evidence presented in this study as well as that reported for cultured granulose cells [10] suggest that either LDL or HDL supports steroidogenesis in ovarian cells, although not to the same extent. For example, if one expresses the results on the basis of the ratio of cholesterol to protein, then HDL seems to be the preferred lipoprotein. However, assuming that 1 mol of human LDL contains $1 \cdot 10^6$ g of cholesterol and each mol of human HDL contains $3.7 \cdot 10^4$ g of cholesterol [10,47] then, on a molar basis, LDL seems to be more effective in providing substrate for ovarian steroidogenesis. Similarly, in cultured granulosa cells on a molar basis, the concentration of HDL required to produce 50% inhibition in [^{14}C]acetate incorporation into cellular free sterols was shown to be 200-fold higher than the concentration of LDL required to produce similar inhibition. Although in the present studies we have investigated the mechanism(s) by which lipoprotein-bound cholesterol is delivered into the cells, previously it has been shown that porcine granulosa cells [48] and bovine granulosa cells [49] possess specific receptors for HDL and LDL, respectively. Studies are currently in progress to delineate whether luteal cells obtain cholesterol from lipoprotein by a receptor-mediated process and, if so, whether HDL and LDL have specific receptors or share a common receptor site on the cell surface.

In summary, the present studies demonstrate the ability of sera from different sources to stimulate steroidogenesis by delivering cholesterol (substrate) into luteal cells in the form of HDL and LDL. Further, luteal cells were found to have an acute requirement for exogenous cholesterol source in the form of plasma lipoproteins for the maximum expression of steroidogenesis.

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