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Ovarian Progesterone Levels During in vitro Oocyte Maturation and Ovulation in Xenopus laevis

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ABSTRACT

Endogenous progesterone was measured in ovaries of the frog *Xenopus laevis* following the administration of gonadotropins that induce meiotic maturation and ovulation of large oocytes. Ovarian pieces were incubated for 0, 1, 3, 5, or 10 h in Gurdon's solution in the presence or absence of human chorionic gonadotropin (HCG; 20 IU/ml) or a frog pituitary homogenate (FPH; 0.04 pituitary/ml). Each incubation sample was scored for ovulation and maturation at the end of its incubation period, homogenized in the medium, and extracted for progesterone with petroleum ether. Following purification on Sephadex LH-20, extracts were assayed for progesterone by a radioimmunoassay that was validated for use with frog ovarian tissue. In 13 experiments, mean (± SEM) progesterone concentration in untreated ovarian tissue was 3.7 ± 0.5 ng/gm at time zero and progesterone levels did not change significantly during 10 h of incubation. HCG-treated tissue (n = 6) exhibited a linear, two-fold increase in progesterone for the first 3 h of incubation and maintained that level for the remainder of the incubation. FPH treatment (n = 10) produced a linear six-fold increase in progesterone over the course of 10 h. Mean progesterone content across time was significantly greater ($P<0.005$) in FPH-treated tissue than in HCG-treated tissue. Both differed significantly ($P<0.05$) from untreated controls. In tissue treated with HCG the percent oocyte maturation was correlated ($P<0.10$) with both mean progesterone concentration across time and progesterone concentration at 10 h of incubation; ovulation did not occur. In FPH-treated tissue progesterone concentration was not correlated with percent maturation but was correlated ($P<0.05$) with the number of ovulations/gm. Treatment of ovarian pieces with the gonadotropins following a 10 h incubation in Gurdon's solution produced effects similar to those following treatment at time zero. *In vivo* treatment with HCG prior to *in vitro* incubation of ovarian tissue with HCG or FPH resulted in an eight- or eleven-fold increase, respectively, in peak progesterone concentration as compared to tissue that was not pretreated *in vivo*. In two experiments large, preovulatory follicles contained sufficiently more ($P<0.05$) progesterone following FPH treatment than did smaller follicles. Both large and small FPH-treated follicles had significantly higher levels of progesterone than untreated control tissue.

INTRODUCTION

The events of meiotic maturation in amphibians are similar to those in most vertebrates. They include movement of the germinal vesicle to a position just beneath the animal pole, breakdown of the nuclear membrane, resumption of meiosis, and arrest of meiosis at second metaphase (Subtelny et al., 1968; Smith and Ecker, 1970). Both gonadotropic and steroid hormones can stimulate meiotic maturation and ovulation of anuran oocytes *in vivo* (Zwarenstein, 1937; Rugh, 1937; Thornton and Evennett, 1969). Application of either gonadotropins or steroids to *Rana pipiens* follicles incubated *in vitro* caused oocyte maturation and ovulation (Schuetz, 1971). When follicle cells were removed progesterone was still effective in inducing maturation but gonadotropins had no effect (Masui, 1967; Thornton and Evennett, 1969). It has been suggested that a gonadotropin stimulates the follicle cells to produce a steroid, which, in turn, acts directly on the oocyte and follicular envelope to cause maturation and ovulation (Wright, 1961; Masui, 1967).

Recent evidence indicated that progesterone might be the endogenous steroid involved in these two processes. When cyanoketone, an inhibitor of the conversion of pregnenolone to progesterone, was added to gonadotropin-treated *Rana pipiens* follicles incubated *in vitro*, ovulation (Wright, 1971) and maturation (Snyder and Schuetz, 1973) were inhibited. The inhibition could be overcome by addition

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of exogenous progesterone. To date only one attempt has been made to measure endogenous progesterone in an amphibian. Chieffi and Lupo (1963) reported a concentration of 16 ng/gm in ovaries of *Bufo vulgaris* during the breeding season. This paper presents the results of the first measurements of ovarian progesterone in amphibians during the course of *in vitro* gonadotropin-stimulated ovulation and maturation.

MATERIALS AND METHODS

Animals and Incubations

Sexually mature *Xenopus laevis* (the African clawed frog) females were killed by decapitation. The ovaries were removed, rinsed in Gurdon's solution (pH 7.6; Gurdon, 1968), weighed, and cut into small pieces of about 35 mg. Pieces chosen at random were weighed and placed in small plastic petri dishes (35 × 10 mm) containing the incubation medium (Gurdon's solution). Total weight of ovarian pieces in one incubation dish ranged from 250–350 mg. When large follicles (stage VI; Dumont, 1972) were incubated separately from smaller follicles, the stage VI follicles were dissected with watchmakers forceps from ovarian pieces totaling 275–340 mg per replicate and were placed in separate incubation dishes from the remaining smaller follicles. Incubation dishes received one of three treatments: 1) Gurdon's solution (control), 2) human chorionic gonadotropin (HCG; Antuitrin S, Parke-Davis)–20 IU/ml, 3) frog pituitary homogenate (FPH)–0.02 *Xenopus laevis* pituitary plus 0.02 *Rana pipiens* pituitary/ml incubation medium. Gonadotropin doses chosen were found in preliminary studies to be optimal for stimulation of ovulation and/or maturation. In each experiment tissues in duplicate or triplicate incubation dishes were treated for 0, 1, 3, 5, or 10 h at 22–25°C.

Near the end of each incubation period, ovulation was scored by gently shaking each ovarian piece and counting the number of oocytes that fell free into the dish. Percent maturation was determined by counting the number of oocytes exhibiting Roux' spot (a white spot that appears at the oocyte animal pole after meiotic maturation) and expressing this number as a percent of the total number of large, preovulatory oocytes (stage VI; Dumont, 1972) with visible animal poles. Maturation scores for dishes in which ovulation occurred were adjusted to account for the number of ovulated mature oocytes whose animal poles would not have been visible had they remained within the ovarian pieces.

Extraction and Purification of Samples

At the end of the incubation period, the contents of each dish were homogenized in the medium. An internal standard consisting of about 2000 counts of [1, 2, 6, 7-^{3}H]-progesterone (New England Nuclear) in 0.1 percent gelatin-0.1M phosphate buffered saline (pH 7; deVilla et al., 1972) was added to each homogenate and the homogenates were extracted with 25 ml petroleum ether (30–60°C) for 45 min on an Eberbach laboratory shaker. Extraction vials were centrifuged at 1100 × g for 30 min and placed at –40°C until the aqueous phase was frozen. The petroleum ether was decanted, the extract evaporated to dryness, and the tubes rinsed twice with petroleum ether to concentrate the extract at the bottom of the tube. Each extract was chromatographed on Sephadex LH-20 (Pharmacia Fine Chemicals, Inc.) using hexane:benzene:methanol (80:5:10) as solvent to separate progesterone from other steroids (Carr et al., 1971) and to remove lipid substances that interfered with measurement of progesterone by radioimmunoassay or competitive protein binding assay. Redistilled or reagent grade solvents were used throughout.

Assay of Progesterone

Following column chromatography, each progesterone fraction was dried and the tubes rinsed twice with petroleum ether. Duplicate aliquots of each sample were taken for radioimmunoassay (RIA) of progesterone and an aliquot was counted for recovery of the added internal standard. Each aliquot for RIA was evaporated to dryness and 100 μl [1, 2, 6, 7-^{3}H]-progesterone (4.6 ng/ml; SA = 96 Ci/mM; New England Nuclear) in 0.1 percent gelatin-0.1M phosphate buffered saline was added to each tube and the tubes vortexed briefly. Then 100 μl of anti-progesterone serum (deVilla et al., 1972), diluted 1:3000 in 0.1 percent gelatin-0.1M phosphate buffered saline, was added to each tube and the rack of tubes was shaken gently by hand and placed at 4°C for 10–24 h. Bound was separated from free progesterone by addition of 1 ml of a dextran-charcoal solution (Echternkamp and Hansel, 1973) to each tube. Racks of tubes were shaken gently by hand, incubated at 4°C for 15 min, and centrifuged at 4°C for 15 min at 1100 × g. The supernatants were decanted into scintillation vials and quantified for tritium. Three replicates of progesterone standards (12.5–1600 pg) were included in each assay. The specificity of the anti-progesterone serum has been tested by deVilla et al. (1972).

In some experiments samples were assayed by both the RIA and a competitive protein binding assay (PBA). The PBA was performed according to the method of Concannon et al. (1975).

Progesterone values are reported as mean (± SEM) "radioimmunoassayable progesterone"/gm tissue and are corrected for recovery. Mean recovery of the internal standard per assay ranged from 49.5–71.4 percent (m = 61.2 percent, SEM = 1.3 percent). Between assay coefficients of variation for two pools of ovarian tissue were 0.15 (n = 7) and 0.21 (n = 10).

Statistical Methods

Significant differences were determined by Student's t test when two means were compared and by analysis of variance for comparisons of more than two means. Scheffé's method for pairwise comparisons was used for making pairwise comparisons between more than two means (Scheffé, 1953).

RESULTS

Validation of Progesterone Radioimmunoassay

The progesterone RIA was validated by several methods for use with *Xenopus* ovarian

TABLE 1. Recovery of cold progesterone added to purified ovarian extracts.

Progesterone added (pg)[a]	Progesterone recovered (pg)[b]	
	Mean ± SEM	%
200	195.2 ± 14.1	98.0
400	402.1 ± 45.3	101.0
800	734.7 ± 60.2	92.0

[a] n = 6, 6, 5.

[b] Corrected for progesterone in sample by subtraction of average pg progesterone measured in samples without added cold progesterone.

extracts. Addition of progesterone (200, 400, or 800 pg) to extracts of untreated frog ovarian tissue demonstrated that progesterone could be accurately measured in the presence of an extract of 31.6 mg of *Xenopus* ovarian tissue (Table 1). Seven assays of duplicate extracts of 15.8, 31.6, and 63.2 mg of untreated ovarian tissue yielded an inhibition curve parallel to that for progesterone standards (Fig. 1). Samples (n = 51) measured for progesterone by both the RIA and the PBA showed a correlation of 0.84 ($Y = 3.1 + 0.9x$, $P<0.005$).

Effect of Gonadotropins on Ovarian Progesterone Concentration

In 13 separate experiments, ovarian tissue from 13 females was incubated in Gurdon's solution alone or in Gurdon's solution plus HCG or FPH. In three of these experiments tissue was exposed to both Gurdon's solution alone and Gurdon's solution plus HCG. In seven experiments tissue was incubated in Gurdon's solution and in Gurdon's solution plus FPH. In three experiments ovarian pieces were incubated in Gurdon's solution alone, in Gurdon's solution plus HCG, and in Gurdon's solution plus FPH.

Progesterone concentrations following incubation in Gurdon's solution, HCG, or FPH are summarized for all 13 experiments in Fig. 2. Mean progesterone concentration at time zero was 3.7 ± 0.5 ng/gm. Progesterone concentration did not change ($P>0.10$) over the course of 10 h of incubation. HCG caused a two-fold increase ($P<0.05$, n = 6) in progesterone during the first three hours of incubation. No further increases were noted up to 10 h. FPH produced a linear increase in progesterone concentration throughout 10 h of incubation, resulting in a six-fold increase ($P<0.005$, n = 10) over control levels. Mean progesterone concentration across time was significantly greater ($P<0.005$) in FPH-treated tissue than in HCG-treated or control tissue. In three experiments in which ovarian pieces were exposed to Gurdon's solution, to HCG, and to FPH, progesterone con-

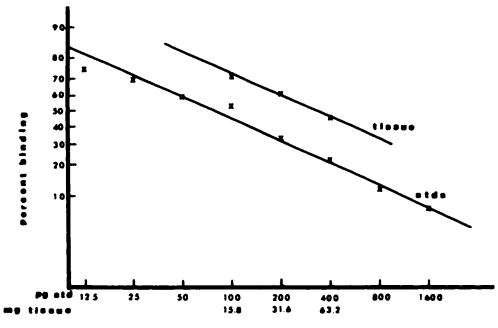

FIG. 1. Percent binding obtained in radioimmunoassays of standard solutions of progesterone or varying amounts of ovarian extracts (n = 7).

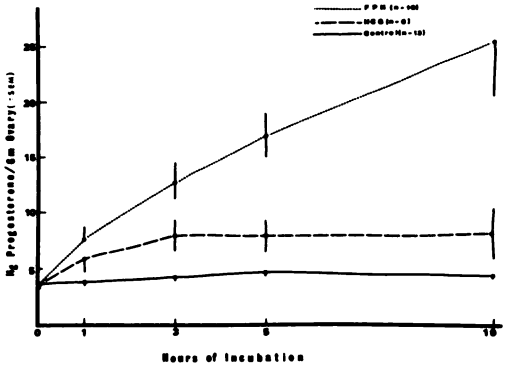

FIG. 2. Average progesterone concentration (ng/gm ± SEM) for all experiments in which ovarian pieces were incubated in Gurdon's solution (control, n = 13) or HCG (n = 6) or FPH (n = 10) for 0, 1, 3, 5, or 10 h.

TABLE 2. Mean percent maturation and mean ovulations/gm ovary induced by HCG and FPH in ovarian tissue incubated *in vitro* (± SEM).

Hormone treatment	Hours of incubation	Mean % maturation (± SEM)	Mean ovulations/ gm (± SEM)
HCG (n = 6)	3	0	0
	5	12 ± 6	0
	10	46 ± 14	0
FPH (n = 10)	3	3 ± 2	0
	5	23 ± 7	1 ± 1
	10	54 ± 7	45 ± 17

centration in FPH-stimulated tissue was significantly higher ($P<0.05$) at 5 and 10 h of incubation than in HCG-stimulated tissue (Fig. 3).

Effect of Gonadotropins on Oocyte Maturation and Ovulation

Ovulation never occurred in ovarian pieces incubated in Gurdon's solution alone for up to 10 h and maturation was observed in only two oocytes. HCG produced an average percent maturation of 46 ± 14 (n = 6). FPH treatment resulted in a mean percent maturation of 54 ± 7 (n = 10). The difference was not significant ($P>0.10$). While HCG treatment never stimulated ovulation in these experiments, FPH treatment resulted in 45 ± 17 ovulations/gm ovarian tissue (Table 2).

Percent maturation was correlated ($P<0.10$) with mean progesterone concentration across time and with progesterone concentration at 10 h in HCG-treated tissue (Table 3). There was no correlation ($P>0.10$) between percent maturation and mean, maximum (peak), or 10 h progesterone concentration for FPH-treated tissue. However, the number of ovulations after FPH treatment was correlated ($P<0.05$) with mean, maximum, and 10 h progesterone concentration (Table 3).

In Vitro Response following In Vivo HCG Treatment

One female was injected four times with 250–300 IU HCG over a period of two weeks. Following each injection the female deposited

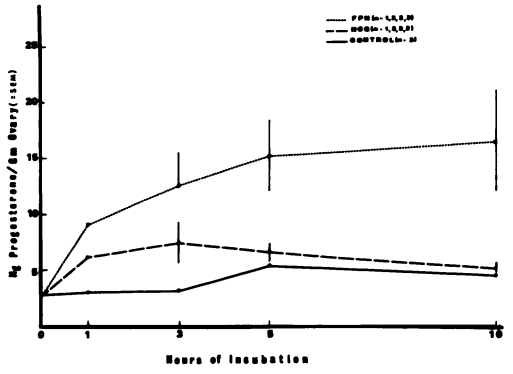

FIG. 3. Average progesterone concentration (ng/gm ± SEM) in ovarian tissue from three females incubated for 0, 1, 3, 5, or 10 h in Gurdon's solution (control) or HCG or FPH.

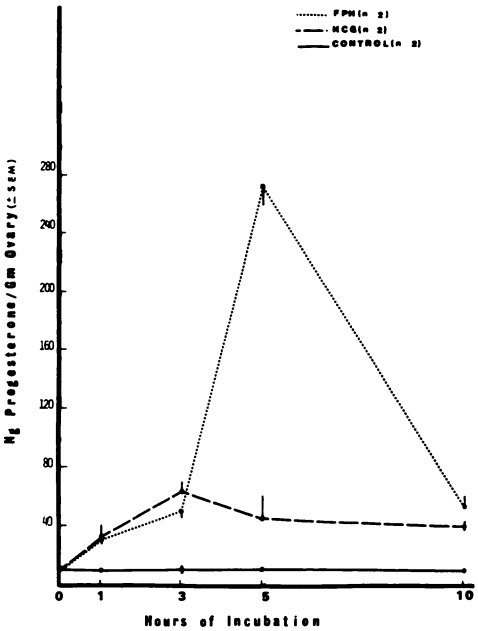

FIG. 4. Progesterone concentration (ng/gm ± SEM) in ovarian pieces from one female incubated for 0, 1, 3, 5, or 10 h in Gurdon's solution (control) or HCG or FPH following HCG treatment *in vivo*.

TABLE 3. Correlation of percent maturation and ovulations/gm with progesterone concentration.

Hormone	Y	Progesterone concentration (X)		
		Maximum	10 hour	Mean
HCG (n = 6)	% Maturation	0.55	0.75[a]	0.71[a]
FPH (n = 10)	% Maturation	0.21	0.23	0.21
FPH (n = 10)	Ovulations/gm	0.71[b]	0.70[b]	0.61[b]

[a] $P<0.10$

[b] $P<0.05$

eggs in the tank. Five days after the last injection, her ovaries were removed and pieces incubated *in vitro* in Gurdon's solution, FPH, or HCG. Peak progesterone levels following treatment with HCG or FPH (Fig. 4) were 8 and 11 times higher, respectively, than in incubations using material from uninjected animals (Fig. 2). The number of ovulations per gram was 25.8, 59.9, and 113.2 in FPH-treated tissue and 6.1, 22.5, and 32.6 in HCG-treated tissue at 3, 5, and 10 h of incubation, respectively.

Progesterone Content of Large vs. Smaller Follicles

Two experiments were conducted to determine whether the increase in progesterone concentration that accompanied gonadotropin-induced ovulation and maturation was associated with the follicles capable of these events. Large, preovulatory follicles (stage VI; Dumont, 1972) were dissected from ovarian pieces and incubated separately from the remaining smaller follicles. Individual incubation dishes contained a mean of 146 (SEM = 4) large follicles; smaller follicles were not counted. Follicles were exposed to FPH and progesterone concentration, percent maturation, and ovulations/gm tissue were determined at 0, 7, and 10 h of incubation. Large and small follicles did not differ significantly ($P>0.10$) in initial progesterone concentration (Fig. 5). Both large and small follicles had more ($P<0.05$) progesterone than untreated controls at 7 and 10 h of incubation. However, the concentration in large follicles was significantly greater ($P<0.05$) than that in smaller follicles at both times. Large follicles contained 6.1, 29.9, and 17.3 pg progesterone (SEM = 3.3) per follicle at 0, 7, and 10 h of incubation, respectively. No maturations were observed in smaller follicles; large follicles exhibited 10 percent and 8 percent maturation at 7 and 10 h, respectively.

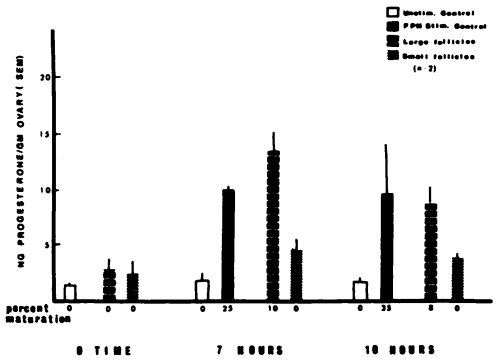

FIG. 5. Progesterone concentration (ng/gm ± SEM) in large follicles, smaller follicles, and intact ovarian pieces taken from two females and incubated in the presence or absence of FPH for 0, 1, or 7 h.

Gonadotropin Treatment following 10 Hours' Pretreatment in Gurdon's Solution

In two experiments ovarian pieces were maintained in Gurdon's solution alone for 10 h and then exposed to FPH or HCG for an additional 10 h. The progesterone concentrations, percent maturation, and number of ovulations/gm differed little from those of other tissue taken from the same two animals and exposed to gonadotropin at time 0 and assayed after 10 h (Table 4).

DISCUSSION

In experiments performed by other investigators, exogenous progesterone provoked maturation, and sometimes ovulation, of amphibian oocytes in the absence of gonadotropins (Zwarenstein, 1937; Schuetz, 1971; Smith and Ecker, 1971). We can conclude on the basis of these

TABLE 4. Progesterone concentration, percent maturation, and ovulations per gram in ovarian pieces 10 hours after hormonal stimulation.[a]

Female	Hormone treatment	Time of stimulation	Progesterone (ng/gm ± SEM)	% Maturation (± SEM)	Ovulations/gm (± SEM)
K	Control	0	4.9 ± 3.4	0	0
	Control	10	4.7 ± 0.8	0	0
	FPH	0	57.1 ± 4.1	48 ± 3	89.8
	FPH	10	68.8 ± 9.4	40 ± 3	75.5
P	Control	0	4.8 ± 0.1	0	0
	FPH	0	12.2 ± 1.0	70 ± 1	45.7
	FPH	10	9.7 ± 2.2	70 ± 1	110.4
	HCG	0	5.2 ± 1.5	45 ± 2	0
	HCG	10	4.8 ± 0.2	54 ± 11	0

[a]Stimulation occurred after 0 or 10 hours incubation in Gurdon's solution.

reports and the results of our experiments that the amphibian gonadotropin induces maturation and ovulation by stimulating an increase in endogenous progesterone. Levels of progesterone measured in gonadotropin-stimulated *Xenopus* ovaries were of the same order of magnitude as those measured by ultraviolet spectrography in ovaries of *Bufo vulgaris* (16 ng/gm; Chieffi and Lupo, 1963). Since gonadotropin treatment following a 10 h incubation in Gurdon's solution produced the same effects as treatment at time 0 (Table 4), it appears that the capacity of ovarian pieces to respond to HCG or FPH did not change over the course of a 10 h incubation. Whether progesterone itself or a metabolite of progesterone was the effective molecule is not known. Sanyal and Sibre (1973) and Reynhout and Smith (1973) found that *Rana pipiens* ovaries metabolized exogenous progesterone to several 5α-reduced derivatives that were also effective in inducing maturation.

FPH-treated tissue contained significantly higher levels of progesterone than did HCG-treated tissue (Fig. 2). This was not due to variation in the response of different animals since the difference between the two gonadotropins was also evident when the two treatments (HCG and FPH) were applied separately to tissue from the same animal (Fig. 3). In addition, FPH provoked ovulation as well as maturation, while HCG induced only maturation. Although mean percent maturation did not differ for the two hormones, percent maturation was correlated with progesterone levels in HCG-treated tissue, while progesterone levels observed following FPH treatment were correlated with the number of ovulations obtained, but not with percent maturation. These correlations suggest that the higher levels of progesterone that accompanied ovulation caused ovulation. This possibility was explored in another series of experiments which will be presented in a subsequent report.

The higher progesterone concentrations observed *in vitro* following HCG treatment *in vivo* (Fig. 4) suggest a relationship between *in vitro* responsiveness and prior gonadotropin exposure. This requires further investigation.

Large follicles contained more progesterone (per unit weight) after FPH treatment than did smaller follicles (Fig. 5), suggesting that the large, pre-ovulatory follicles are the major site of progesterone synthesis in gonadotropin-stimulated tissue. However, the smaller follicles contained significantly more progesterone than untreated tissue. The role of progesterone in the smaller follicles remains to be studied. Throughout oogenesis amphibian oocytes are surrounded by a single layer of follicle cells (Franchi, 1962). So the higher levels of progesterone produced by large *Xenopus* follicles cannot be attributed to a larger number of follicle cells (per unit weight).

In conclusion, the results presented here demonstrate that *Xenopus* ovaries contain and synthesize progesterone and provide the first direct evidence that gonadotropins induce ovulation and maturation of amphibian oocytes by stimulating an increase in ovarian progesterone concentration. The data suggest that ovulation requires higher threshold levels of progesterone than does maturation.

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