Differential inhibition by progesterone of the adenylate cyclase of oocytes and follicle cells of *Xenopus laevis*

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Received 13 April 1982

1. INTRODUCTION

Progesterone can induce the meiotic maturation of full-grown *Xenopus laevis* oocytes under well-defined culture conditions. The action of this hormone on amphibian oocytes differs from the mechanism established for steroid hormones in other systems since progesterone acts on the external membrane of the oocytes and does not require the presence of the oocyte nucleus to initiate the maturation process (review [1]).

Reports from several groups have suggested that the hormonal induction of maturation is related to a transient decrease in intracellular cAMP observed in oocytes treated with progesterone [2–5]. In [6–8] progesterone was shown to inhibit in vitro the adenylate cyclase of full-grown oocytes from *Xenopus laevis*. In addition, progesterone treatmet of oocytes reduced the formation of cAMP from ATP microinjected into these cells [9]. The in vitro effect on adenylate cyclase was specific for the steroid analogs that can trigger maturation; the inhibitory effect was observed at hormone concentrations required to produce oocyte maturation.

How specific is a steroid hormone of this inhibition with respect to the target cells of progesterone? Here we address the question with regard to the different cells of the *Xenopus laevis* ovary. The results show that progesterone inhibits the adenylate cyclase present in oocytes at different stages of development but does not affect the enzyme of the follicle cells.

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2. MATERIALS AND METHODS

Adult *Xenopus laevis* females were obtained from South African Snake Farm (Capetown) and were maintained with constant 12 h light and dark periods at 22°C. Their ovaries were obtained as in [10].

[α-^{32}P]ATP was prepared using [^{32}P]phosphate from Amersham [11]. Sigma supplied unlabeled ATP which was purified [12] and beef heart cyclic nucleotide phosphodiesterase.

2.1. *Preparation of follicle cells and defolliculated oocytes*

Manual method: Whole follicles consisting of full-grown oocytes (1.2–1.3 mm diam.) surrounded by a layer of follicle cells were separated from the ovary by means of watchmaker forceps. These follicles were incubated in a solution containing 116 mM NaCl, 1.4 mM KCl, 10 mM Tris–HCl (pH 7.6) and 1 mM EDTA as in [13] for 2 h at 25°C with mild shaking. The complete layer of follicle cells, which is loosened by this procedure, was removed intact with forceps under the dissecting microscope and was returned together with the defolliculated oocytes to isotonic saline at 22°C. Microscopic examination of stained sections of oocytes defolliculated by this method showed that < 5% of the follicle cell layer remained attached to the oocytes.

Collagenase defolliculation method: Small pieces of ovary were incubated in amphibian saline with 2 mg collagenase/ml (Sigma, type II) for 3 h at 25°C with mild stirring essentially as in [14]. This procedure dissociates the tissue into oocytes that are almost completely follicle-free. The cells of different diameters may then be separated by sieving and leaves a suspension of the follicle cell layers. The

Published by Elsevier Biomedical Press
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oocytes were passed over a 700 μm mesh nylon screen (Nitex). Oocytes retained on this screen were mainly stages IV, V and VI according to [15] and were further separated manually into stage IV (700–900 μm) and stages V and VI (1000–1300 μm). Oocytes which passed through the 700 μm screen were further fractionated using 500 μm and 53 μm Nitex screens, followd by hand selection to give stages III, IV and stages I–II. Loosened follicle cells which sediment very slowly as compared to the intact oocytes were removed and concentrated from the mother liquors by centrifugation at 1000 × *g* for 10 min. Again, microscopic examination of the defolliculated oocytes of different stages showed that these cells were 95% follicle free.

2.2. *Preparation of oocyte and follicle cell homogenates and particulate adenylate cyclase*

Defolliculated oocytes were homogenized in 1 vol. solution containing 0.88 M sucrose in buffer A (50 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol and 1 mM EDTA) with a Dounce homogenizer using 10 strokes each of pestle A and B. The homogenate was diluted to 0.22 M sucrose in buffer B (as A but pH 7.5) and a 105 000 × *g* particulate fraction was prepared by differential centrifugation as in [8].

Follicle cells obtained after collagenase treatment of ovary as above were homogenized in 10 vol. 0.88 M sucrose in buffer B and processed as given for oocytes except 0.44 M sucrose was used for centrifugations. The fraction sedimenting at 20 000 × *g* which contained > 80% of the adenylate cyclase activity was used for characterization studies.

2.3. *Adenylate cyclase activity*

Membrane fractions and homogenates were assayed for adenylate cyclase activity essentially as in [8]. The final reaction mixture contained in 100 μl, 50 mM Tris–HCl (pH 7.5), 1 mM cAMP, 1 mM dithiothreitol, 0.5 mM EDTA, 5% glycerol, 5 mM $MgCl_2$ or $MnCl_2$, 4 mM creatine phosphate, 40 μg creatine kinase, 40–105 μM [α-^{32}P]ATP, (250–3900 cpm/pmol), and 2–200 μg protein of the enzyme preparations. NaF and quanyl-5′-yl-imidodiphosphate (Gpp(NH)p) were added as indicated. Assays were performed at 32°C or 37°C in triplicate and at several levels of protein to insure proportionality to activity. In those experiments in which progesterone was added to the reaction mixture, a control was performed in the absence of hormone but with added solvent, 0.06% ethanol. Cyclic [^{32}P]AMP formed was analyzed as in [8,10] and corrected for the recovery of added c[^{3}H]AMP. In addition, all analyses include a correction for any radioactivity remaining after incubation of the product c[^{32}P]-AMP in the presence of bovine heart cAMP phosphodiesterase, prior to the fractionation on Dowex-50 and alumina columns.

3. RESULTS

Recent reports have described the properties of adenylate cyclase of ovarian follicles and of defolliculated oocytes of *X. laevis* ovary [8,10]. To study the specificity of the progesterone inhibition, however, it seemed important to differentiate between the enzyme of the oocyte itself and that of the surrounding follicle cell layer. The homogenates of the follicle cell layers contained adenylate cyclase activity equivalent in amount to the enzyme found in an equal number of full-grown defolliculated oocytes.

Table 1 shows that the adenylate cyclase present in the total homogenate of the follicle cells responds to stimulation by Gpp(NH)p and by NaF, indicating that this enzyme is similar to the oocyte activity and has the G/F regulatory subunit described for

Table 1

The effect of Gpp(NH)p and fluoride ion on adenylate cyclase activity of follicle cells of *X. laevis* ovary

	Follicle cell adenylate cyclase activity (pmol/mg protein 15 min)		
	Basal	+ Gpp(NH)p (0.1 mM)	+ Fluoride (10 mM)
$MnCl_2$ (5 mM)	292	996	4056
$MgCl_2$ (5 mM)	102	574	2508

160 full-grown stage VI follicles were defolliculated manually and the follicle cell layers homogenized in 300 μl buffer B and 0.44 M sucrose. Three levels of homogenate protein (1.3–5.3 μg) were assayed under standard conditions at 37°C in triplicate, with the additions indicated. The values presented are averages of those obtained with linear dependence on the amount of enzyme added [α-^{32}P]ATP was 0.07 mM (1900 cpm/pmol)

other systems [16]. The stimulation by both effectors is seen with Mg^{2+} or Mn^{2+}. Although all activities are higher in the presence of Mn^{2+}, Mg^{2+} was used throughout the rest of the experiments because this ion is required for the physiologically regulated activity of adenylate cyclase in other tissues [16] and in oocytes [18].

Differential centrifugation of the follicle cell homogenate indicated that 82% of the cyclase activity is present in the 20 000 × *g* sedimented fraction with only 12% and 6% being present in the high speed pellet and in the soluble fraction, respectively. The scant activity present in the soluble fraction, however, was not responsive to NaF or guanine nucleotides.

Fig.1A shows the effect of different concentrations of progesterone on the adenylate cyclase activity of the follicle cell preparation and that of the full-grown defolliculated oocytes from the same ovary. Since the action of the hormone requires the presence of guanine nucleotides [8], both cyclase preparations were assayed in the presence of 25 μM Gpp(NH)p. Different responses are observed with the two preparations in that the oocyte enzyme is inhibited 50% by 1 μM progesterone whereas the follicle cell enzyme is not affected even at 10 μM progesterone. The steroid hormone concentrations that are required for the induction of maturation of these oocytes are between 0.1–1 μM. The adenylate cyclase activities found in the high speed pellet and soluble fractions of the follicle cell homogenates likewise are not inhibited by progesterone. Also progesterone does not affect the initial lag of the reaction catalyzed by the adenylate cyclase of the follicle cells in the presence of Gpp(NH)p (not shown).

The ovary of *Xenopus laevis* contains oocytes at different stages of development which can be defined by size and morphology [15]. In [10] the adenylate cyclase activity of whole follicles containing oocytes was reported at different stages of oogenesis, but the sensitivity of the enzyme to progesterone was only tested for full-grown oocytes.

Table 2 presents the analysis of the effect of 1 μM progesterone on the adenylate cyclase activity obtained from defolliculated oocytes at different stages of development. It is evident that the adenylate cyclase present in all the different oocyte stages can be significantly inhibited by progesterone. However, the extent of inhibition obtained with the

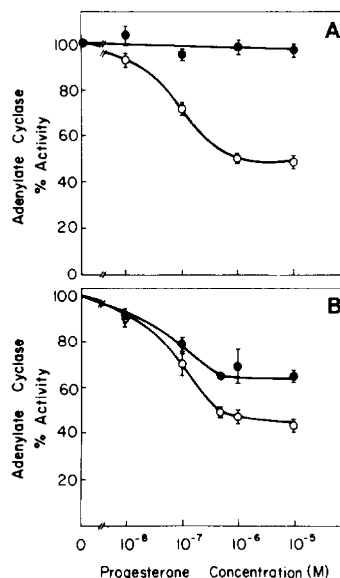

Fig.1. The effect of progesterone concentration on the relative activity of adenylate cyclase from different ovarian cells. Bars indicate standard deviations.

(A) Follicle cells and full-grown defolliculated oocytes were separated by the collagenase method and homogenates were prepared and fractionated as in section 2. The adenylate cyclase activity present in the 20 000 × *g* pellet fraction of follicle cells (●——●) and in the 105 000 × *g* pellet fraction of full-grown oocytes (○——○) was assayed in the presence of 5 mM $MgCl_2$, 0.06% ethanol, 25 μM Gpp(NH)p, 0.1 mM [α-^{32}P]ATP (240 cpm/pmol) and the indicated amount of progesterone, incubating 20 min at 32°C as in section 2. The 100% activity for the follicle cells was 1009 pmol/mg protein when 8 μg protein was used and for the full-grown oocytes the 100% value was 69.7 pmol/mg protein when 170 μg protein was used.

(B) Pre-vitellogenic (stages I and II) oocytes and full-grown oocytes (stage VI) were defolliculated an separated by the collagenase treatment in section 2. Homogenization and subsequent fractionation was done as in section 2 except that for the stage I and II oocytes the centrifugation at 20 000 × *g* was omitted and therefore the particulate fraction used as enzyme source contained both the intermediate and high speed pellets. Aliquots of the enzymes for the stage I and II (●——●) and stage VI (○——○) oocytes were assayed in triplicate in the presence of 5 mM $MgCl_2$, 0.06% ethanol, 25 μM Gpp(NH)p, 0.1 mM ATP (750 cpm/pmol) and of the amounts of progesterone indicated, incubating for 20 min at 32°C (section 2). The 100% activity value of the stage I and II oocytes was 20.3 pmol cAMP synthesized/mg protein when 285 μg protein were used per assay and the 100% value of the stage VI oocytes was 60.8 pmol cAMP synthesized/mg protein when 230 μg protein were used per assay.

Table 2

Effect of progesterone on adenylate cyclase activity of different stage oocytes from *X. laevis* ovary

Oocyte stage	Adenylate cyclase activity (pmol cAMP/mg protein)		
	Control	+ Progesterone (1 μM)	Inhibition (%)
I, II	3.7 (0.1)	2.6 (0.3)	30
III	9.0 (0.3)	6.1 (0.4)	32
IV	12.7 (0.4)	9.7 (1.4)	24
V, VI	34.5 (3.4)	17.2 (0.8)	50

Different stage oocytes were sized after collagenase treatment and the 105 000 × *g* particulate fractions prepared as in section 2. Assays were performed at 32°C for 20 min with 5 mM $MgCl_2$, 25 μM Gpp(NH)p with 0.10 mM [α-^{32}p]ATP (1160 cpm/pmol) with or without 1 μM progesterone, and the remainder as in section 2. Numbers in parentheses give the standard deviations of the values obtained. The significance of the inhibition for each class of oocytes is: $p < 0.0025$ for stages I and II; $p < 0.01$ for stage III; $p < 0.012$ for stage IV; and $p < 0.0005$ for stages V and VI

small and medium size oocytes (30%) has been consistently smaller than the inhibition observed with full-grown cells (50–60%). Fig.1B shows the effect of different concentrations of progesterone on the adenylate cyclase of pre-vitellogenic oocytes (stages I and II) and of full-grown cells (stage VI) from the same ovary. It seems clear that the hormonal concentrations required for enzyme inhibition are similar but that the extent of maximal inhibition is considerably greater for the larger cells.

4. DISCUSSION

The above results demonstrate clearly that the inhibitory effect of progesterone on adenylate cyclase is specific for the enzyme present in the oocytes of the ovarian tissue of *Xenopus laevis*. Progesterone has no effect on the adenylate cyclase of the follicle cells, although the enzyme activity obtained from these cells is similar to that found in oocyte membranes both in amount and in its capacity to be stimulated by NaF and Gpp(NH)p. That inhibition of adenylate cyclase by progesterone is restricted to certain target cells of the ovarian tissue adds physiological relevance to the observation and suggests that there may also be specificity in the distribution of the progesterone receptor molecules that must participate in the effect. A membrane protein has been found to have the properties of a progesterone receptor in *X. laevis* oocytes [17].

However, the demonstration that the adenylate cyclase from oocytes at early stages of oogenesis can also be inhibited by progesterone indicates that the triggering of maturation is a complex phenomenon. Since small and medium size oocytes (stages I–V) do not mature in the presence of progesterone under standard culture conditions or in animals treated with hormone, it seems clear that the capacity of the adenylate cyclase to be inhibited by progesterone is not sufficient per se to make these cells responsive to the hormone in undergoing meiotic maturation. In this respect, the lower inhibition of the adenylate cyclase observed in the unresponsive oocytes may be important and it may be postulated that a threshold level of inhibition is required to trigger the process. Also pertinent to the complexity of the process is the observation that the maturation of mammalian oocytes seems to be under negative regulation by an inhibitor present in the follicular fluid [18]. Similar mechanisms may be present in the smaller amphibian oocytes as shown by the fact that stage V oocytes become responsive to progesterone if they are defolliculated [19] and that stages IV and V oocytes exposed for long periods to potassium-free media can also be induced to mature by progesterone [20]. The two latter observations support the conclusion, which can likewise be drawn from the above data, that the smaller oocytes have progesterone receptors.

ACKNOWLEDGEMENTS

This work was supported by grants from The Ford Foundation, UNDP/Unesco project CHI/81/001, the Organization of American States and the University of Chile.

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