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Effect of Defolliculation and 17α -Hydroxy, 20β -Dihydroprogesterone on Cyclic AMP Level in Full-Grown Oocytes of the Rainbow Trout, *Salmo gairdneri*

B. Finet, B. Jalabert, and S.K. Garg

Institut National de la Recherche Agronomique, Laboratoire de Physiologie des Poissons, Campus de Beaulieu, Rennes, France (B.F., B.J.); Department of Zoology, Haryana Agricultural University, Hissar, India (S.K.G.)

The changes in cAMP were followed in trout oocytes incubated in vitro after defolliculation performed by either enzymatic or manual dissection. Both defolliculation methods induced a highly significant rise in oocyte cAMP level (4.5 times the basal level of control [follicle-enclosed oocytes], after 6 h). Treatment of defolliculated oocytes with 17α -hydroxy, 20β -dihydroprogesterone ($17\alpha, 20\beta$ -OH-P) (10^{-6} M), which induced oocyte maturation (germinal vesicle breakdown [GVBD]) was able, first, to interrupt the increase of oocyte cAMP level promoted by defolliculation and then to lower this level significantly down to values that still remained higher than folliculated controls. Very low concentrations of $17\alpha, 20\beta$ -OH-P (1.38 – 55.6×10^{-9} M), or physiological doses of testosterone (0.35×10^{-6} M, in the range found in vivo before ovulation) were able to induce a similar decrease of oocyte cAMP level without inducing GVBD. Under the same experimental conditions estradiol (0.35×10^{-6} M) exhibited no action. These results suggest that some factor(s) originating in the follicle (FIF), inhibit the oocytes' tendency to accumulate cAMP before the final surge of $17\alpha, 20\beta$ -OH-P. This factor might be a follicular steroid such as testosterone or nonmaturing concentrations of $17\alpha, 20\beta$ -OH-P. Moreover our data favour the hypothesis that the final surge of $17\alpha, 20\beta$ -OH-P could induce distinct intraoocyte mechanisms: the first induces an irreversible blockage of cAMP level before the inhibitory action of the FIF is suppressed by ovulation, and the second mechanism leads to GVBD.

Key words: oocyte maturation, cyclic AMP, fish

INTRODUCTION

Since the first report on the efficiency of 17α -hydroxy, 20β -dihydroprogesterone ($17\alpha, 20\beta$ -OH-P) to induce oocyte maturation (resumption of meiosis in fully grown oocytes) in vitro in trout follicles [Fostier et al., 1973], converging data have allowed us to consider this steroid to be the principal physiological maturation-inducing steroid

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Address reprint requests to Dr. B. Finet, Institut National de la Recherche Agronomique, Laboratoire de Physiologie des Poissons, Campus de Beaulieu, 35042 Rennes Cedex, France.

(MIS) in trout [Jalabert and Finet, 1986] and probably in all salmonoids [Nagahama et al., 1983; Nagahama and Adachi, 1985]. From the little experimental evidence available so far in trout, some features related to the possible mechanism of action of $17\alpha,20\beta$ -OH-P on trout oocytes are more or less similar to that of progesterone in *Xenopus* [reviewed by Maller, 1985], i.e., the post-transcriptional character of maturational events following action of the MIS [Jalabert, 1976] and the implication of cAMP in the regulation of some of these events [Goetz and Hennessy, 1984; Jalabert and Finet, 1986].

In *Xenopus*, an early action of progesterone is the inhibition of oocyte plasma membrane adenylate cyclase [Mulner et al., 1979; Jordana et al., 1981; Finidori-Lepicard et al., 1981; Sadler and Maller, 1981], resulting in a transient decrease of total oocyte cAMP [Cicirelli and Smith, 1985], which is the beginning of a cascade of cellular events leading to germinal vesicle breakdown (GVBD) [Maller, 1983; Ozon, 1983]. However, the relative importance of this phenomenon has been controversial, and the hypothesis that cAMP is not the only early intracellular messenger involved in oocyte maturation following the action of progesterone has been recently advanced by Bellé et al. [1986] and Chien et al. [1986]. In trout, in addition to indirect evidence resulting from the maturation-inhibiting action of different substances that induce a rise in intraoocyte cAMP, a significant decrease of oocyte cAMP was observed in vitro following the action of $17\alpha,20\beta$ -OH-P [Jalabert and Finet, 1986]. This decrease, however, was of low magnitude and exhibited different kinetics, depending on the experiment, making it difficult to define precisely the phenomenon in the chronology of maturation induction. One reason for this variability could have been the methodology employed, which involved the incubation of intrafollicular oocytes and cAMP extraction after crushing the whole follicles and thereafter centrifugation to separate the oocyte contents from the follicular envelopes.

The aim of the present work was to follow more precisely the evolution of cAMP after treatment with $17\alpha,20\beta$ -OH-P in trout oocytes devoid of their follicular envelopes, to define more precisely the chronology of cAMP response (if any), and to evaluate in a better way the role of cAMP fluctuations in the mechanism of action of $17\alpha,20\beta$ -OH-P in oocyte maturation.

MATERIALS AND METHODS

Animals

Female rainbow trouts, 2 or 3 years old weighing from 0.9 kg (first-time spawners) to 1.8 kg (second time spawners), were acclimated in recirculated water units (natural photoperiod, temperature regulated between 10 and 14°C). During the expected "spawning period" all the fish were anaesthetized (2-phenoxy-ethanol, 0.3‰ in water) at weekly intervals in order to check the ovarian stage, determined by observation of a few follicles squeezed out by abdominal stripping [Jalabert and Fostier, 1984].

Mediums for Dissection and Incubation

Dissection (DM 8/280) and incubation media (IM 8/280) were prepared according to Jalabert and Finet [1986] and adjusted to pH 8. Osmotic pressure was adjusted to 280 mosmol.

Oocyte Preparation

Females exhibiting oocytes at the subperipheral or peripheral germinal vesicle stage were killed, and the ovaries were removed and dissected in DM 8/280, at 4°C, into small clusters of two to six follicles.

Oocyte Defolliculation

Enzymatic defolliculation. Defolliculated oocytes were obtained by enzymatic treatment (Jalabert and Heydorff, unpublished): batches of 600 follicles were incubated in 1 liter Erlenmeyer flasks containing 20 ml of defolliculation medium (DFM) and submitted to gentle rotative agitation for about 10 h. DFM was prepared with IM 8/280 containing in addition bovine serum albumin (BSA), 2 mg/ml; polyvinylpyrrolidone (Av. Mol. Wt.: 40,000), 2 mg/ml; dextran-sulfate, 1 mg/ml; hyaluronic acid, 0.05 mg/ml; epinephrine-bitartrate, 10 µg/ml; and collagenase, 0.32 mg/g of oocyte. At the end of incubation, defolliculated oocytes were thoroughly washed with DM 8/280 at 4°C and selected under a stereomicroscope.

Manual defolliculation. Manual defolliculation could be achieved by exerting a moderate pressure with a small glass cuplet in order to create a little budding on the follicle wall. Ovarian epithelium, theca folliculi, and some granulosa cells could then be removed as a single layer, using watchmakers forceps.

Incubation

Follicles and manually or enzymatically defolliculated oocytes, were preincubated in IM 8/280 (five or six oocytes/ml), at 12°C for 2 h, under weak agitation. Incubations with hormones were performed under the same conditions. The rate of maturation was scored as the percentage of germinal vesicle breakdown (GVBD) 48 h or 72 h after hormonal treatment, to ascertain the possibility of a delay in the maturational process.

Oocyte Extract and cAMP Assay

The following steps were carried out at 0–4°C. Groups of five oocytes (when incubations were performed with follicle-enclosed oocytes, these were manually denuded just before cAMP extraction) were quickly homogenized using a Potter-Elvehjem tissue grinder in a 10-ml solution prepared with 5 ml 0.4 M HCl mixed with 5 ml 0.02 M ethylenediamine tetracetic acid (EDTA)/2 mM theophylline to block phosphodiesterase activity. The tubes were centrifuged for 10 min at 4,000g. Further steps for cAMP extraction and cAMP assay were adapted from Meurs et al. [1980] as has already been described by Jalabert and Finet [1986]. The binding protein was isolated from rabbit skeletal muscle purified as described by Gilman [1970] and modified by Tovey et al. [1974].

Scanning Electron Microscopy

Oocytes were obtained by enzymatic or manual defolliculation and processed as follows. A first fixation step was performed by a 3-h immersion in 2.5% solution of glutaraldehyde in vehicle buffer (stock solution of glutaraldehyde 25% was first rectified to pH 7.4 according to Chambers et al. [1968]). The vehicle buffer contained 0.1 M sodium cacodylate and sucrose to reach 320 mosmol). After a 1-h rinsing in vehicle buffer, a second fixation step was performed by a 1-h immersion in 1% solution of osmium tetroxide in vehicle buffer. Oocytes were then dehydrated in

acetone according to Perry [1984] and kept in 100% acetone. The oocytes were observed using a scanning microscope (Jeol JSM 35) after processing in a critical-point drier.

Chemicals

Steroids were purchased from steraloids; BSA, polyvinylpyrrolidone (PVP 40), dextran-sulfate, heparine, hyaluronic acid, theophylline, and adenosine 3':5'-cyclic monophosphate (cAMP) were obtained from Sigma. Tritiated cAMP (1.09 TBq/mmol) was from Amersham (France), and collagenase was from Boehringer Mannheim (France).

Statistical Analysis

All experimental points represented in figures are the mean of three values, each one being the median value of a triplicate cAMP assay carried out on the extract from a sample of five oocytes. Treatments and experiments were compared either by Student's t-test or by one- and two-way analysis of variance (time-treatment or treatment-female). Differences between groups were estimated by Student's t-test.

RESULTS

Defolliculation

The aspect of oocyte surface after enzymatic or manual defolliculation observed by SEM is shown in Figure 1. All the enzyme-treated oocytes were completely devoid of granulosa cells (G.C.). However some remnants of microvilli, still inserted in pores of the zona radiata, were observed. A representative region of the whole surface of a typical enzyme-treated oocyte is shown in Figure 1A.

As shown in Figure 1B manually treated oocytes were only partially defolliculated. The oocyte surface was covered either by small plaques of G.C. or by a carpet of residual microvilli.

Effect of Defolliculation on cAMP Content of Oocytes Incubated In Vitro

Fluctuations of cAMP content were followed after enzymatic or manual defolliculation in oocytes from nine different females. Large variations were found in the initial cAMP content of oocytes from different females and in the relative magnitude of changes induced by defolliculation. Therefore, as these changes were similar from one female to the other, the typical changes in oocytes from only one female are presented in Figure 2. In all cases defolliculation induced a greater and increasing accumulation of cAMP, sometimes significant even at the first time point of the kinetics (i.e., after one night in DFM [enzymatically defolliculated oocytes] or in IM8-280 [manually defolliculated oocytes] and 2 h of preincubation). Considering the nine experiments, the intraoocyte cAMP concentrations after 6 h of incubation were about 4.5 times higher in defolliculated oocytes than in oocytes incubated within their follicles. Significant differences were observed in some experiments between manually and enzymatically defolliculated oocytes, cAMP levels remaining lower in manually defolliculated oocytes than in enzymatically treated ones (Fig. 3).

Effect of Steroids on Defolliculated Oocytes

Effect of continuous exposure of defolliculated oocytes to $17\alpha, 20\beta$ -OH-P. Continuous exposure of defolliculated oocytes to high doses of $17\alpha, 20\beta$ -OH-P in-

Fig. 1. Scanning electron micrographs of rainbow trout oocytes. **A:** Enzymatically defolliculated oocyte showing some microvilli (v) inserted in pores of zona radiata ($\times 4000$). **B:** Manually defolliculated oocyte with granulosa cells (g) on the surface of zona radiata ($\times 200$).

Fig. 2. Changes in oocyte cAMP content as a function of the time of incubation of manually (○) or enzymatically (●) defolliculated oocytes or of intrafollicular oocytes (△). The figure presents a typical experiment. Each point represents the mean cAMP contents ($\pm$ SD) of three samples of five oocytes. Zero-time point refers to the 0-Time cAMP determination. Significant differences between treatments: (S★★) $P < .01$.

duced meiotic maturation in manually or enzymatically defolliculated oocytes with a small loss of sensitivity in some cases (Jalabert and Heydorff, unpublished). Two experiments were scheduled to look for the possible occurrence of cAMP fluctuations within 1 h after the addition of $17\alpha, 20\beta$ -OH-P (oocyte samplings were done after 0.5, 1, 2, 4, 10, 15, 30, and 60 min). Since no significant variations in cAMP levels could be detected during this short interval (data not shown), nine other experiments were performed over a period of 18 h. Under these experimental conditions, treatment of enzymatically or manually defolliculated oocytes with $17\alpha, 20\beta$ -OH-P (10^{-6} M), lowered cAMP levels significantly, from 2 h to the end of the experiment (Fig. 3), and induced meiotic maturation (GVBD) in spite of the fact that cAMP levels were still significantly higher than in the nonmaturing control oocytes (i.e., oocytes incubated within their follicle, without the addition of $17\alpha, 20\beta$ -OH-P). When treatments on either enzymatically or manually defolliculated oocytes were compared, similar decreases of about 40% of oocyte cAMP content were observed after 6 h of incubation.

Effect of short exposure of defolliculated oocytes to $17\alpha, 20\beta$ -OH-P. An exposure of 3 or 15 min to an excess of $17\alpha, 20\beta$ -OH-P ($3 \cdot 10^{-6}$ M) was sufficient to induce GVBD in enzymatically defolliculated oocytes. No significant differences were observed in the decrease of cAMP levels in oocytes that were exposed to $17\alpha, 20\beta$ -OH-P ($3 \cdot 10^{-6}$ M) for 3 or 15 min or were kept continuously in the steroid (Fig. 4). Low levels of cAMP were maintained during 18 h of incubation, even after a brief exposure of 3 min to $17\alpha, 20\beta$ -OH-P.

Effect of different concentrations of $17\alpha, 20\beta$ -OH-P administered continuously. Very low concentrations of $17\alpha, 20\beta$ -OH-P (1.38 – $55.6 \cdot 10^{-9}$ M) (Fig. 5), which were unable to promote meiotic maturation in defolliculated oocytes, maintained oocyte cAMP at low levels, which were not significantly different from cAMP

Fig. 3. Effect of $17\alpha,20\beta$ -OH-P on cAMP accumulation in enzymatically defolliculated oocytes (solid lines) and manually defolliculated oocytes (dotted lines). Defolliculated oocytes were incubated with (solid symbols) or without (open symbols) $17\alpha,20\beta$ -OH-P (10^{-6} M). Control oocytes were incubated within their follicle and were defolliculated just before cAMP extraction (Δ). Zero-time refers to the addition of $17\alpha,20\beta$ -OH-P. In that typical experiment results are the mean ($\pm$ SD) of three experimental points assayed in triplicate. Significant differences between treatments: $P < .01$ (S**); no significant difference: NS.

Fig. 4. Effect of duration of exposure of defolliculated oocytes to the action of $17\alpha,20\beta$ -OH-P ($3 \cdot 10^{-6}$ M). Enzymatically defolliculated oocytes were incubated 3 min ($\bullet$), 15 min ($\blacksquare$), or 18 h ($\blacktriangle$) with an excess of $17\alpha,20\beta$ -OH-P ($3 \cdot 10^{-6}$ M), then extensively washed with DM8/280, and incubated in IM8/280 during 48 h for scoring the rate of maturation. Defolliculated oocytes ($\square$), intrafollicular oocytes (Δ) incubated without hormone. Mean ($\pm$ SD) cAMP contents of three samples of five oocytes.

Fig. 5. Effect of different concentrations of $17\alpha,20\beta$ -OH-P on cAMP accumulation and germinal vesicle breakdown (GVBD) of enzymatically defolliculated oocytes. Oocytes cAMP content is expressed as % ($\pm$ SEM) of control without hormone. Cyclic AMP was extracted after 6 h of incubation. GVBD was estimated after 48 h of incubation. Significant differences between treatments: $P < .001$ (S***); no significant difference: NS. The numbers on the top of the bars represent the number of individual experiments.

values noticed in oocytes induced to mature by higher concentrations of $17\alpha,20\beta$ -OH-P. No proportionality was ever observed between concentrations of $17\alpha,20\beta$ -OH-P, the rate of cAMP decrease (compared to control), and the rate of induction of maturation.

Effect of nonmaturing steroids. Testosterone added to the incubation medium at two different concentrations, $0.35 \cdot 10^{-6}$ M (Fig. 5) or $3.5 \cdot 10^{-6}$ M (data not shown), was able to decrease significantly cAMP levels in defolliculated oocytes. This decrease of cAMP levels was of the same magnitude as obtained after treatment of oocytes with $17\alpha,20\beta$ -OH-P, but was not followed by GVBD. In contrast, estradiol 17β (Fig. 6) exhibited no action either on cAMP levels or on oocyte maturation.

DISCUSSION

The results presented here concerning the effect of defolliculation demonstrate that oocytes that were enzymatically devoid of their follicles exhibited a large accumulation of cAMP. Since proteolytic enzymes are known to be able to stimulate adenylate cyclase in cellular systems [Hanoune et al., 1982], similar experiments were conducted using manually defolliculated oocytes. The fact that manual defolliculation induces a similar increase in cAMP levels to that observed in enzymatically treated oocytes suggests that defolliculation itself could be responsible for the modification of oocyte cAMP concentrations. Although we cannot exclude the possibility of a direct action on adenylate cyclase activity of endogenous proteolytic enzymes released by the damaged follicular cells during manual defolliculation, an attractive

Fig. 6. Effect of $17\alpha,20\beta$ -OH-P (P) ($0.3 \cdot 10^{-6}$ M), testosterone (T) ($0.35 \cdot 10^{-6}$ M) or estradiol- 17β (E) ($0.35 \cdot 10^{-6}$ M) on oocyte cAMP content, after 6 h of incubation with hormones, in enzymatically defolliculated oocytes. Values are expressed in % ($\pm$ SEM) of control without hormone. The numbers on the top of the bars represent the number of individual experiments. Significant differences between treatments: $P < .001$ (S***).

hypothesis would be that the removal of follicular layers suppresses the action of a putative follicular-inhibiting factor (FIF), produced by the follicular cells and acting on the oocyte cAMP levels. The oocyte cAMP increase noticed during the end of the follicular growth [Jalabert and Finet, 1986] could be related with a decreasing effect of the FIF. Moreover, the observation that cAMP levels were sometimes higher in enzymatically defolliculated oocytes than in manually defolliculated ones could be explained by the fact that, in contrast to enzymatically defolliculated oocytes, manually defolliculated oocytes were not totally devoid of their follicular cells, the amount of remnant granulosa cells depending on the donor female.

The rise of trout oocyte cAMP observed here after defolliculation appears original, contrasting with observations in amphibians in which cAMP levels remain unchanged after defolliculation [Maller and Krebs, 1980], and in some mammals in which an opposite phenomenon has been reported, spontaneous meiotic maturation induced by defolliculation being preceded by a fall in intraoocyte cAMP levels [Schultz et al., 1983; Aberdam et al., 1987]. The mechanisms involved in this decrease of cAMP still remain controversial, but one hypothesis is that a maturation-inhibitory factor, originating from follicle cells, may be involved in the regulation of oocyte cAMP level [Schultz et al., 1983; Thibault and Gerard, 1987]. To our knowledge the work presented here is the first description of a follicular negative control on cAMP level in fish oocytes.

Concerning the effect of meiotic-inducing steroid (MIS) on defolliculated oocytes, our data demonstrate that meiotic maturation promoted by $17\alpha,20\beta$ -OH-P is preceded by an inhibition of cAMP accumulation induced by defolliculation and by a decrease of intraoocyte cAMP. This effect appears to be irreversible and was not obtained with a nonmaturational steroid such as estradiol. However further experimental observations suggest that this decrease in the cyclic nucleotide concentration is necessary, but not sufficient for inducing meiotic resumption of rainbow trout oocytes.

1. In spite of a decrease in cAMP level preceding meiotic maturation, the level of the cyclic nucleotide remained higher throughout the incubation period than the basal level in nonmaturing controls. These findings are not in agreement with the hypothesis generally advanced in amphibians [Maller, 1983; Ozon, 1983], in which a decrease of oocyte cAMP induced by progesterone leads to a decrease in the cAMP-dependent protein phosphatase(s) of a hypothetical maturation protein (Mp-P), capable in its dephosphorylated form (Mp) of initiating the formation of the autocatalytically amplified oocyte maturation-promoting factor (MPF) [Masui and Markert, 1971; Reynhout and Smith, 1974]. According to this scheme, maturation could occur only when cAMP concentration reaches a threshold level, lower than the basal one, thus inducing a decrease of cAMP-dependent phosphorylation.

2. Very low concentrations of $17\alpha,20\beta$ -OH-P or preovulatory levels [Fostier and Jalabert, 1982; Scott et al., 1983] of testosterone, which do not promote meiotic resumption, decreased cAMP concentrations by the same magnitude as doses of $17\alpha,20\beta$ -OH-P that were sufficiently high to bring GVBD.

These two points favour the preponderance of the hypothesis that the maturational surge of $17\alpha,20\beta$ -OH-P may act simultaneously through the regulation of cAMP level by irreversibly repressing its increase before ovulation (or before defolliculation), and through yet another cellular mechanism to promote oocyte maturation in a dose-dependent manner. The hypothesis of a dual mechanism of control of oocyte maturation, obtained from other kinds of experimental studies, was advanced in *Xenopus* [Blondeau and Beaulieu, 1985], and recently Belle et al. [1986] suggested that in *Xenopus* oocytes, progesterone could activate protein phosphatase(s) involved in Mp-P dephosphorylation through a change in free oocyte Mg^{2+} . Such a hypothesis of a dual route of control could explain our results in trout oocyte maturation as follows:

The observation that $17\alpha,20\beta$ -OH-P is able to induce maturation in spite of oocyte cAMP concentrations higher than the basal levels, might be explained by the fact that this steroid hormone may act through the other route of control (i.e., the phosphatase route), which would compensate an increased activity of cAMP-dependent protein kinase owing to elevated cAMP concentrations.

The fact that very low concentrations of $17\alpha,20\beta$ -OH-P or physiological concentrations of testosterone (a nonmaturational steroid present in the blood and produced in the ovary from the end of vitellogenesis, when estradiol secretion decreases, up to the surge of $17\alpha,20\beta$ -OH-P) are able to maintain low levels of cAMP without inducing meiotic maturation, could be explained by their selective action via the cAMP route, which may not be sufficient for induction of maturation, but might be necessary to keep the whole system equilibrated before the final action of the maturational surge of $17\alpha,20\beta$ -OH-P. In this view, if their action, in contrast to maturing doses of $17\alpha,20\beta$ -OH-P, was not irreversible, they would play the role of the follicular-inhibiting factors.

The findings that very high levels of oocyte cAMP, like those obtained after treatment with forskolin [Jalabert and Finet, 1986], inhibited the maturational process induced by $17\alpha,20\beta$ -OH-P may be explained by the impossibility for the "phosphatase route" to compensate totally the increase of protein kinase activity promoted by abnormally high levels of cAMP. In vivo, the inhibitory follicular factor would maintain the oocyte cAMP concentrations to a level compatible with the induction of the maturation by $17\alpha,20\beta$ -OH-P through the other route of control.

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