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Effects of Inhibitors on Forskolin- and Testosterone-Induced Steroid Production by Preovulatory Medaka Follicles

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ABSTRACT

In follicles of the medaka *Oryzias latipes*, forskolin (F) stimulated production of both estradiol-17β (E_2) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-diOHP) during 20 hr of incubation, while exogenous testosterone stimulated only the former. Effects of actinomycin D, amanitin, cycloheximide and puromycin on the F- and testosterone-induced steroid production were investigated during an *in vitro* culture system for preovulatory ovarian follicles. When isolated preovulatory follicles were incubated in medium containing actinomycin D, amanitin, cycloheximide or puromycin combined with F or with testosterone, F- and testosterone-induced E_2 and 17α,20β-diOHP production was inhibited. A similar inhibitory effect was also observed with cyanoketone, which is a specific inhibitor of 3β-hydroxy-Δ^5-steroid dehydrogenase (3β-HSD). These results suggest that the F- and testosterone-induced E_2 and 17α,20β-diOHP production in medaka ovarian follicles is probably mediated by *de novo* synthesis of mRNA.

Key words : steroid production, cyanoketone, forskolin, protein synthesis, medaka follicle

INTRODUCTION

It has been well established that the meiotic maturation of fish oocytes is under the control of one or more steroid hormones, the maturation-inducing steroid(s). Currently, the steroids are believed to be produced by gonadotropin-stimulated ovarian follicle cells in several teleosts (Fostier *et al*., 1983 ; Nagahama, 1983). However, the mechanism of steroid production is not adequately known in teleosts.

Cyclic AMP seems to have a primary role in mediating the effects of gonadotropin on the enhancement of 17α,20β-dihydroxy-4-pregnen-3-one (17α, 20β-diOHP) production in amago salmon granulosa cells (Nagahama *et al*., 1985), is well established for the effect of gonadotropin on progesterone production in mammals (Catt and Dufau, 1976; Kolena and Channing, 1972; Marsh, 1976; Robinson *et al*., 1971). Forskolin (F), which increases the intrafollicular level of cAMP, has been used as a tool to investigate the role of cAMP in the regulation of meiotic maturation in mammalian oocytes (Racowsky, 1984) and of steroid production (Kwon and Schuetz, 1985; Tan *et al*., 1986). The involvement of protein synthesis in this process is suggested by the fact that cycloheximide inhibits F-induced estradiol-17β (E_2) production. On the other hand, exogenous testosterone (T) stimulates E_2 production in follicles isolated during vitellogenesis (Sakai *et al*., 1988). The mechanism of stimulation by exogenous T is not known for the fish follicles.

The objective of the present study was to determine whether or not protein synthesis is involved in F- and T-induced steroid production *in vitro*. We have found that protein synthesis and mRNA synthesis inhibitors inhibit both F- and T-induced steroid production, and suggest that some steps in the F- and T-induced steroidogenic process required *de novo* protein synthesis and transcription of mRNA.

MATERIALS AND METHODS

Preparation of isolated preovulatory follicles

Adult medakas (*Oryzias latipes*) were purchased from a fish farmer (Koriyama, Nara Pref.) and kept under reproductive conditions (14 hr light, temperature 26-28°C) for more than 2 weeks before use. From females that had spawned every day, ovaries were removed 21 hr and 11 hr before ovulation, which occurred about one hr before the onset of light, and placed into a saline solution (Iwamatsu, 1980). Large preovulatory follicles were isolated by dissecting the ovaries with watchmaker forceps then transferred into fresh culture medium (90% Earle's medium 199, Dainippon-seiyaku, Osaka) supplemented with 30 μg/ml penicillin G-K (Meiji-seika) and 60 μg/ml streptomycin sulfate (Meiji- seika).

Denuded follicles containing oocytes covered with granulosa cells alone without the thecal cell layer and basement membrane were prepared, as described elsewhere (Iwamatsu, 1980).

Incubation of follicles

Isolated follicles were eventually apportioned into the wells of a culture plate (Cell Wells; Corning Glass Works) and placed in an incubator (27°C). In each well, 5, 10 and 20 individual follicles were incubated in 1 ml of medium.

To test the ability of forskolin (F : Calbiochem-Behring) to induce oocyte maturation, large preovulatory follicles were preincubated in medium containing the drug for a given time, then incubated further for 10-18 hr in fresh plain medium. The effects of testosterone (T; Sigma), 2 α-cyano-4, 4, 17α-trimethyl-androst-5-en-17β-ol-3-one (CK, cyanoketone ; Sterling Winthrop), actionomycin D (Sigma), amanitin (Sigma), cycloheximide (Sigma) and puromycin (Sigma) on the F-induced production of E_2 and 17α,20β-diOHP were examined mixing each reagent in culture medium in which follicles were incubated for 20 hr.

Stock solutions of F (1mM), T (10 μg/ml) and CK (100 μg/ml) dissolved in ethanol were used by adding them to the incubation medium to give a final ethanol concentration of less than 1 %.

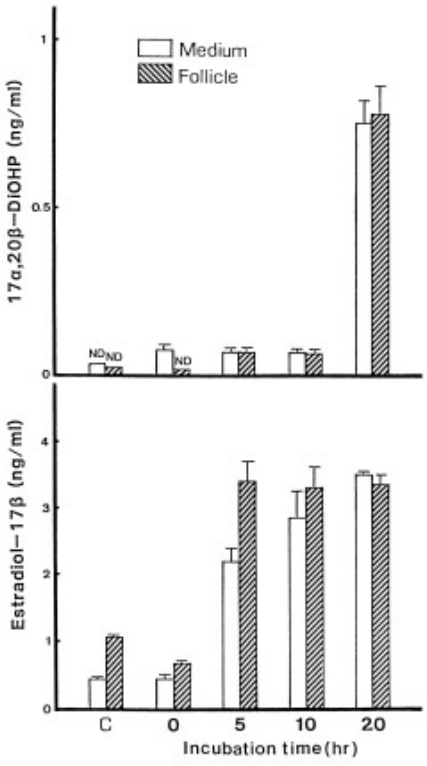

Fig. 1 Effect of forskolin (F) on estradiol-17β and 17α,20β-dihydroxy- 4 -pregnen- 3 -one (17α,20β-diOHP) production by preovulatory medaka follicles. Ten follicles were incubated in 1 ml of medium with or without F (20 μM). The shaded columns and the blank columns indicate the values measured in follicle homogenates and the values in media, respectively. The vertical bars represent the mean ± SEM of the four replicates. C: contol group incubated in plain medium for 20 hr. ND: non-detectable (<30 pg/ml).

Examination of oocyte maturation

At the end of incubation, oocytes were examined for germinal vesicle breakdown (GVBD) and for their ability to develop upon fertilization after ovulation or artificial removal of their follicle layers with fine forceps (Iwamatsu *et al*., 1987).

Radioimmunoassay of steroids

Following incubation, media were collected and stored at −20°C until radioimmunoassay for E_2 (Kagawa *et al*., 1982) and 17α,20β-diOHP (Young *et al*., 1983). Four replicate incubations were performed for each experimental and control group.

Preparation of data

All data were presented as mean ± SEM. The significance of group differences was determined by Student's *t*-test.

RESULTS

Effect of FK on E_2 and 17α,20β-diOHP production by ovarian follicles

As reported previously (Iwamatsu *et al*., 1987), F of submaximal concentrations (10 or 20 μM) stimulated both E_2 and 17α,20β-diOHP production by fully grown (preovulatory) follicles (10/ml) just before stimulation by gonadotropin would

have occurred *in vivo* (21 hr before ovulation). E_2 production began 5 hr after exposure to 20 μM F, and production of 17α,20β-diOHP began between 10 hr and 20 hr (Fig. 1). Control follicles (10/ml) that were incubated in plain medium for 20 hr, produced a small quantity of E_2 (about 1 ng/ml in follicles: about 0.5 ng/ml in medium), but no 17α,20β-diOHP, as reported by Iwamatsu *et al*. (1987) and Sakai *et al*. (1987).

The capacity of follicles (5/ml) to produce E_2 and 17α,20β-diOHP in the presence of 10 μM F was examined in two groups of isolated fully grown follicles at 11 hr and 21 hr before ovulation. When follicles were isolated 21 hr before ovulation and incubated either for the first 10 hr only (from 21 hr to 11 hr before ovulation) or for first 20 hr (from 21 hr to one hr before ovulation) in the continuous presence of F, about 1.8 ng/ml or about 1.0 ng/ml of E_2 was produced respectively. In the follicles that were isolated and incubated for 10 hr beginning 5 hr (11 hr before ovulation) before germinal vesicle breakdown (GVBD) of the oocyte, production of E_2 was no longer stimulated by F (Fig. 2). In this follicle group, which was isolated after *in vivo* stimulation by gonadotropin (release between 3 hr and 10 hr after onset of light: Iwamatsu, 1978), the capacity to produce 17α,20β-diOHP was already presence irrespective of the F-effect. However, follicles isolated from ovaries 21 hr before ovulation were unable to produce 17α,20β-diOHP when incubated in plain medium. When these follicles were exposed to F for only the first 10 hr or for the first 20 hr (from 21 hr to one hr before ovulation), they produced about 0.3 ng/ml or about 1.9 ng/ml of 17α,20β-diOHP, respectively.

Effects of inhibition of mRNA and protein synthesis on forskolin-induced 17α,20β-diOHP and E_2 production

Fully grown follicles (20/ml, 21 hr before ovulation) were incubated in media containing cycloheximide (10 μg/ml), puromycin (0.1, 1 and 10 μg/ml), actinomycin D (0.1, 1 and 10 μg/ml) or amanitin (10 μg/ml), added to 10 μM F. As shown

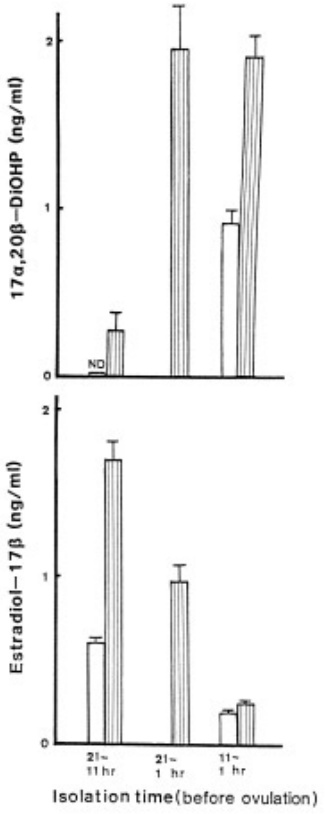

Fig. 2 Effect of forskolin on estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-diOHP) production by preovulatory follicles isolated 11 hr or 21 hr before ovulation. Preovulatory follicles (5/ml) isolated 11 hr or 21 hr before ovulation were incubated with (striped columns) or without (blank columns) forskolin (10 μM) until presumptive ovulation time. ND: non-detectable (<30 pg/ml).

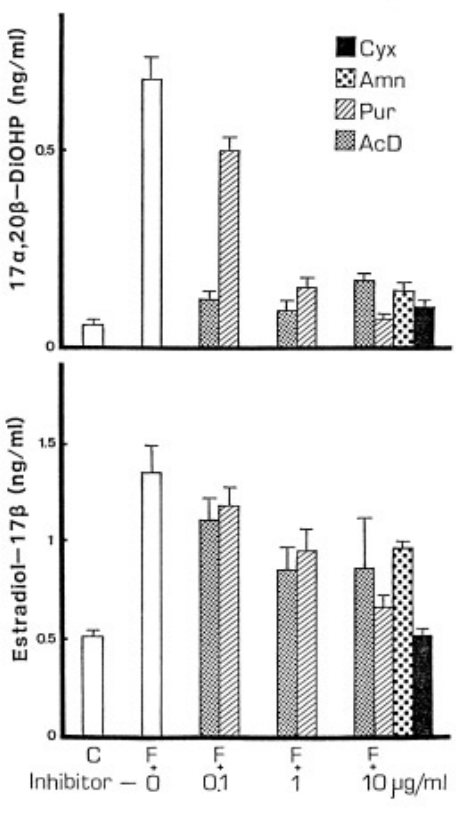

Fig. 3 Effects of cycloheximide, amanitin, puromycin and actinomycin D on forskolin-induced estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-diOHP) production. Twenty preovulatory follicles were incubated in 1 ml of medium containing forskolin (F: 10 μM) combined with (+) each inhibitor for 20 hr. Cyx, cycloheximide; Amn, amanitin; Pur, puromycin; AcD, actinomycin D.

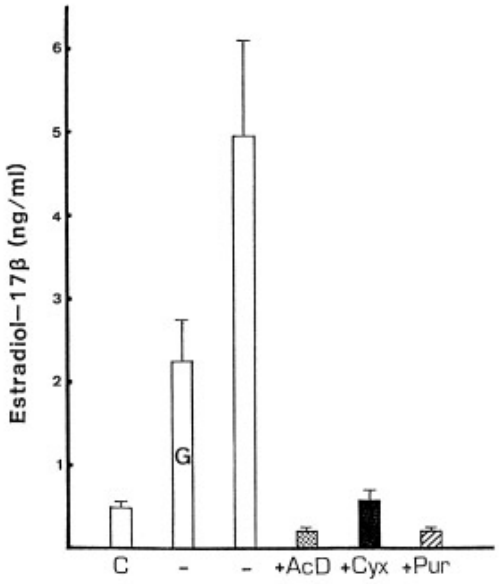

Fig. 4 Effects of actinomycin D, cycloheximide and puromycin on testosterone-induced estradiol-17β production. Five preovulatory follicles except for G group were incubated for 20 hr in 1 ml of medium containing testosterone (1 ng/ml) with (+) or without (−, blank columns) actinomycin D (AcD), cycloheximide (Cyx) or puromycin (Pur). The "G" column indicates follicles (the granulosa cell layer alone surrounding the oocyte) without the thecal cell layer and the basement membrane. C: preovulatory follicles of control group incubated in plain medium.

in Fig. 3, although F alone stimulated follicle cells to produce E_2 (about 1.3 μg/ml) and 17α,20β-diOHP (about 0.7 μg/ml), the F-induced E_2 and 17α,20β-diOHP production was significantly suppressed ($P<0.05$) by actinomycin D (1 and 10 μg/ml), amanitin (10 μg/ml), puromycin (1 and 10 μg/ml) and cycloheximide (10 μg/ml). The inhibitory effect of puromycin was dose-dependent.

Inhibitory effects of inhibitors of mRNA and protein synthesis on testosterone-induced E_2 production

Testosterone (T, 100 ng/ml) stimulated E_2 production by whole follicles and denuded fully grown follicles without the thecal layer and basement membrane (G, marked in column of Fig. 4). T-induced E_2 production by fully grown follicles (5/ml, 21 hr before ovulation) was inhibited by inhibitors of both mRNA (10 μg/ml, actinomycin D)- and protein (10 μg/ml puromycin and 10 μg/ml cycloheximide)- synthesis (Fig. 4).

Effects of cyanoketone and testosterone on forskolin-induced E_2 and 17α,20β-diOHP production

To ascertain the action site of F in production of E_2 and 17α,20β-diOHP, the effects of T (100 ng/ml) and CK (1 μg/ml), a specific inhibitor of the enzyme 3β-hydroxy-Δ^5 steroid dehydrogenase (3β-HSD), were examined. Production of 17α,20β-diOHP by F-stimulated preovulatory follicles (5/ml, 21 hr before ovulation) was completely inhibited in the presence of CK, but it was not affected by exogenous testosterone. On the contrary, E_2 production was stimulated by exogenous T as a precursor, while it was only partially inhibited by CK (Fig. 5). T and F synergistically promoted E_2 production. The F-induced E_2 and 17α,20β-diOHP production was abrogated in the presence of CK. Incubated intrafollicular oocytes matured only in culture media containing F alone, which stimulated production of 17α,20β-diOHP by the follicles. Consequently, the action site of F in production of E_2 seems to mediate the action of 3β-HSD.

Effect of cyanoketone on progesteroneinduced E_2 and 17α,20β-diOHP production

The effect of CK on production of E_2 and 17α,20β-diOHP in follicular cells by addition of progesterone (10-100 ng/ml) was examined in fully grown follicles. In the presence of CK (1 μg/ml), the progesterone-induced production of both E_2 and 17α,20β-diOHP was not inhibited (Fig. 6); this was significantly higher ($P<0.01$) than that in the absence of CK by mechanism unknown at present. Incubated intrafollicular oocytes matured in culture media containing 100 μg/ml of progesterone, which induced production of 17α,20β-diOHP by the follicles.

DISCUSSION

The present study found that F-induced production of E_2 and 17α,20β-diOHP by preovulatory medaka follicles was inhibited in the presence of inhibitors of protein synthesis such as cycloheximide and puromycin. This suggests that in the medaka, production of E_2 and 17α,20β-diOHP by preovulatory follicles in response to F-stimulation involves *de novo* synthesis of protein(s), as presumptively summarized by a diagram (Fig. 7). The F-induced development of cell organs such as rough endoplasmic reticulum and Golgi complexes (Iwamatsu and Ohta, 1989) may also indicate active synthesis of proteins. Since cAMP-induced E_2 and 17α,20β-diOHP production (our unpublished data) as well as stimulation of cAMP production by F (Iwamatsu *et al*., 1987; Takahashi *et al*., 1991) are inhibited by cycloheximide, most of the effects of F may reflect those of cAMP, which induces steroid production dependent on new synthesis of protein(s). The increase in intracellular cAMP induced by salmon GTH or hCG results in elevation of 20β-HSD activity to 17α,20β-diOHP (Nagahama, 1994). Similarly, it has been reported that 17α-hydroxy-progesterone and 17α,20β-diOHP production induced by F and dbcAMP in amago salmon ovarian follicles (Nagahama *et al*., 1985) and E_2 production by goldfish ovarian follicles (Tan *et al*., 1986) in response to gonadotropin are blocked by cycloheximide (Nagahama *et al*., 1991; Senthilkumaran *et al*., 2004). F induced both E_2 and 17α,20β-diOHP production, but only its effect on production of these steroids is abolished of mRNA synthesis. This suggests

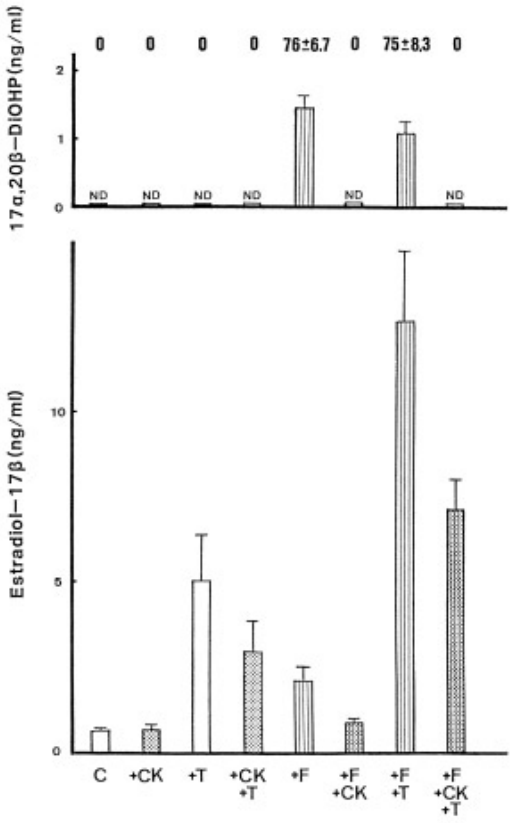

Fig. 5 Effects of cyanoketone and testosterone on forskolin-induced production of estradiol-17β and 17α, 20β-dihydroxy-4-pregnen-3-one. Preovulatory follicles were incubated in media containing cyanoketone (CK) combinated with testosterone (T), in addition to forskolin (F). Numbers at the top of the columns indicate the percentage of oocyte maturation at the end of incubation.

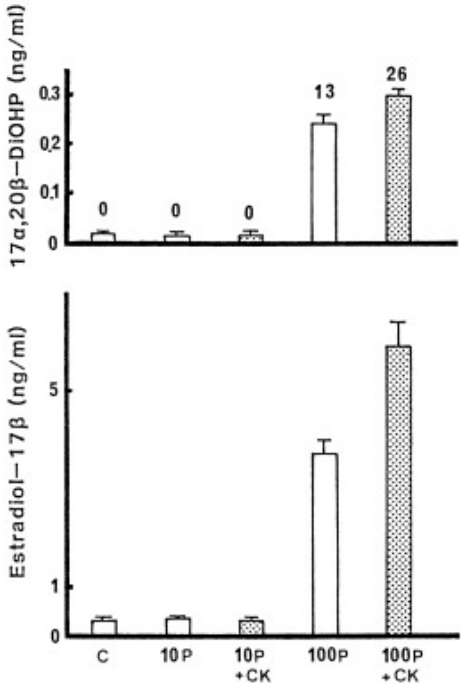

Fig. 6 Effect of cyanoketone on progesterone-induced production of estradiol-17β and 17α, 20β-dihydroxy-4-pregnen-3-one. Preovulatory follicles (5/ml) incubated in media containing cyanoketone (CK) combined with progesterone (P ; 10 and 100 ng/ml) for 20 hr. Numbers at the top of the columns indicate the percentage of oocyte maturation at the end of incubation.

that both mRNA synthesis and protein synthesis are involved in the F-induced synthesis of 17α,20β-diOHP. This agrees with the suggestion that gonadotropin acts on granulosa cells to enhance the *de novo* synthesis of 20β-HSD by a mechanism involving RNA synthesis (Nagahama *et al*., 1985). Thus, F appears to exert its effects on 17α,20β-diOHP production at the level of gene transcription for the 20β-HSD protein.

According to previous studies (Sakai *et al*., 1987; Iwamatsu *et al*., 1988), ovarian follicles of the medaka isolated during vitellogenesis are capable of producing E_2 in response to gonadotropin (GTH) as the follicle increases in size. 3β-HSD mRNA levels in pre-vitellogenic or early vitellogenic follicles were elevated after incubation with LH and/or a cAMP agonist in rainbow trout (Young *et al*., 1999), and aromatase mRNA levels increased in brown trout early vitellogenic follicles in response to FSH *in vitro* (Montserrat *et al*., 2004). But the ability of follicle to produce E_2 upon GTH stimulation ceases during a critical period at the end of vitellogenesis (between 11 and 15 hr before ovulation). The present data also reveal the poor ability of post-vitellogenic follicles to produce E_2 in response to F. Our previous reports suggested that the induction of E_2 production (probably aromatase activity) is stimulated with increase in the intrafollicular cAMP by F (Iwamatsu *et al*., 1987) and a serum factor (Iwamatsu *et al*., 1988).

As shown in the present data, the 3β-HSD inhibitor CK abrogates the stimulatory effect of F on 17α,20β-diOHP and E_2 production by preovulatory medaka follicles. This result agrees with the report that 3β-HSD is essential for stimulation by F of E_2 production in goldfish ovarian follicles (Tan *et al*., 1986). CK also inhibits *in vitro* maturation of intrafollicular medaka oocytes induced by GTH stimulation (Iwamatsu and Onitake, 1983). Thus, F seems to stimulate E_2 and 17α,20β-diOHP production *via* conversion of pregnenolone to progesterone.

In the medaka, aromatase activity, assessed indirectly by the conversion of exogenous testosterone to E_2, increased during vitellogenesis with the maximal activity in follicles 20 hr before spawning (Sakai *et al*., 1988). This coincides with the present

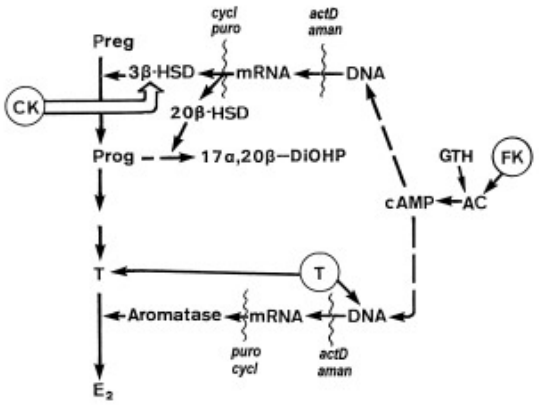

Fig. 7 Hypothetical pathways of steroids in the ovarian follicle of the medaka. The production of cAMP is enhanced by gonadotropin (GTH) and forskolin (FK), and then estradiol-17β (E_2) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-diOHP) are produced *via de novo* protein synthesis. This pathway is suppressed by cyanoketone (CK). However, CK does fail to suppress production of E_2 enhanced by exogenous testosterone.

data in follicles 21 hr before ovulation (about 22 hr before spawning). The maximal ability to produce E_2 in preovulatory follicles synchronizes with that stimulated by exogenous testosterone (T). On the other hand, exogenous T stimulated E_2 production but did not stimulate 17α,20β-diOHP and was only partially affected by CK. The stimulation of T to produce E_2 was inhibited by inhibitors of mRNA and protein synthesis. These suggest that exogenous T stimulates E_2 production not only as a precursor of E_2 (Nagahama *et al*., 1991; Tanaka *et al*., 1992; Fukada *et al*., 1996), but also *via* new production of proteins (presumably aromatase or its regulatory protein) by inducing mRNA synthesis, as illustrated in Fig. 7. Recently, the data of Luo and Wiltbank (2006) on bovine granulosa cells have revealed that mRNA for follicle-stimulating hormone receptor and CYP19A1 (aromatase) were increased by exogenous T and dihydrotestosterone.

In post-vitellogenic medaka follicles, E_2 production sharply decreases (Sakai *et al*., 1987). The reduced production of E_2 may be in part due to decreased aromatase activity. This unlike to be, at least, due to the decrease in the production of endogenous androgen such as testosterone, because E_2 production is not improved by addition of testosterone in culture medium (Sakai *et al*., 1988). The mechanisms by which E_2 production in the medaka post-vitellogenic follicles becomes low are still unknown. In the present experiments, F- and T-stimulated E_2 production was inhibited by inhibitors of protein synthesis, suggesting that F- or T-stimulated E_2 production is mediated by new production of protein. Thus, the sharp decrease in E_2 production may be due to inhibition of the newly produced mRNA and/or proteins at the critical post-vitellogenic phase, rather by an inactivation of aromatase.

On the other hand, 17α,20β-diOHP production after the critical post-vitellogenic phase depends on new synthesis of protein *via* mRNA transcription. A shift from production of E_2 to that of 17α,20β-diOHP takes place in the medaka follicle during the critical period of post-vitellogenesis. This may be due to not only to stimulation of synthesis of proteins such as 20β-HSD *via* synthesis of mRNA but also to the decline of synthesis and/or activity of aromatase due to the degeneration of follicle cells.

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