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Roles of Theca and Granulosa Cells in Follicular Steroidogenesis in *Rana dybowskii*

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Previously, we have proposed a two-cell type model for follicular steroidogenesis in amphibians with *Rana nigromaculata*. Present experiments were carried out to ascertain whether the model is applicable to *R. dybowskii*. The role of theca layer were also reassessed by using granulosa cell-free pure theca layer (P-THEP). Theca/epithelium (THEP) layers, P-THEP layers, and granulosa cell enclosed-oocytes (GCEOs) were obtained from ovarian follicles of *R. dybowskii* by microdissection. Intact follicles (IFs) and different types of tissues were cultured for 6 hour in amphibian Ringer's in the presence or absence of FPH (0.05 gland/ml) or various steroid precursor (100 ng/ml). The amounts of product steroids converted by the components were measured by RIA. Exogenously added pregnenolone (P_5) resulted in a marked increase in progesterone (P_4) by GCEOs (2143 pg/follicle) and IFs (2346 pg/follicle) but a smaller increase in P_4 by THEP layer (495 pg/follicle). Addition of P_4 increased 17α-hydroxyprogesterone (17α-OHP_4) levels by GCEOs (1118 pg/follicle) and IFs (1333 pg/follicle) but less by THEP layer (290 pg/follicle). However, much less amounts of P_4 or 17α-OHP_4 were produced by P-THEP layers than THEP in the presence of P_5. Exogenous 17α-OHP_4 increased androstenedione (AD) levels by GCEOs (1415 pg/follicle) and IFs (561 pg/follicle) but not by THEP layers. In contrast, addition of AD resulted in a marked increase in testosterone (T) levels by THEP (2594 pg/follicle) and IFs (2223 pg/follicle) but much less by GCEOs (339 pg/follicle). Exogenous T increased estradiol (E_2) levels by GCEOs (551 pg/follicle) and IFs (887 pg/follicle), but not by THEP layer (<10 pg/follicle). Without addition of FPH or steroid precursors, very low or nondetectable levels of steroids were produced (< 20 pg/follicle) by all the types of follicular components examined. The data presented here indicate that the two-cell type model based on the study with *R. nigromaculata* is applicable to *R. dybowskii* and also suggest that the minor pathway, which convert P_5 to 17α-OHP_4, is not present in theca layer.

KEY WORDS: Amphibians, Frog, Steroidogenesis, Ovarian Follicles

It is well established that growth and maturation of amphibian oocytes are regulated by steroids produced by ovarian follicles in response to gonadotropins (Masui and Clarke, 1979; Schuetz, 1985). Several reports indicated that follicle walls are responsible for steroidogenesis in amphibians (Thibier-Fouchet *et al.*, 1976; Mulner *et al.*, 1978; Schuetz and Lessman, 1982). The follicle walls are consisted of three major somatic cell layers; a surface epithelium, an outer theca layer,

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and an inner granulosa cell layer (Masui, 1967; Schuetz 1974). However, there are only a limited informations about the relative roles of the different type of cell layers in the follicular steroidogenesis in amphibians. Granulosa cells were known to produce progesterone (P_4) in response to gonadotropin and to convert 25-OH-cholesterol to P_4 in *Rana pipiens* follicles (Schuetz and Lessman, 1982; Petrino and Schuetz, 1987). Using *R. nigromaculata* follicles, we also examined the relative roles of theca and granulosa cells in follicular steroidogenesis and proposed two-cell type model in which granulosa cells are the main sites for production of P_4, 17α-OHP_4, AD and E_2, and theca/epithelium (THEP) layers are those for T (Kwon and Ahn, 1994). However, the two-cell type model was based on the data from only one species of *Rana* and it was unclear whether theca layers can convert P_5 to 17α-OHP_4 since the thecal layer used in the former studies turned out to be partially mixed with granulosa cells. Thus, present experiments were carried out to ascertain whether the two-cell type model is applicable to *R. dybowskii* and to ascertain whether pure theca layer can convert P_5 to 17α-OHP_4.

Materials and Methods

Animals

Most frogs (*R. dybowskii*) were collected from streams in Chonnam area during hibernation periods (October - December). Animals were kept in a state of artificial hibernation in a cold room maintained in full darkness at 4°C. Medium-sized follicles were obtained from frogs collected in August - September. They were kept in plastic boxes containing tap water and maintained at room temperature and were used for experiments within 3 days of collection.

Culture of follicular components

After animals were killed by decapitation, ovaries were removed immediately and divided into several fragments in amphibian Ringer's (AR). Intact follicles (IFs), granulosa cell-enclosed oocytes (GCEOs), theca/epithelium (THEP) layers and granulosa cell free pure THEP layer (P-THEP) were separated from ovarian fragments by manual dissection under a dissecting microscope. The detailed microdissection technique was described by Schuetz and Lessman (1982). Using watchmaker's fine forceps, the THEP layer with blood vessels were peeled away from the follicles in ovarian fragments, and simultaneously the GCEOs were separated from the fragments. Usually, most granulosa cells are remained on the oocyte membrane rather than on THEP layer. However, small number of granulosa cells were found in THEP layers. We obtained pure THEP layer, free of granulosa cells, by peeling off the outside of basal laminar layer from follicle for this experiment.

Different types of follicular components were cultured for 6 hours in AR in the presence or absence of FPH (0.05 gland/ml) or various steroid precursors (P_5, P_4, 17α-OHP_4, AD or T; 100 ng/ml, each). FPH was prepared from female frog which collected at corresponding experimental periods. Glands were homogenized in AR at 4°C using a glass homogenizer. Homogenate was centrifuged (15,000 × g, 20 min, 4°C) to remove debris, and the supernatant was frozen (-20°C) in aliquots until needed. All steroids were purchased from Sigma Chemical Co. (St. Louis, MO). The duration of follicle culture and the doses of FPH or exogenously added steroids were chosen on the basis of our previous data from several species of *Rana* (Ahn *et al.*, 1993; Kwon *et al.*, 1993 1994). In each experiments different types of follicular components were distributed into 24 well tissue culture dishes (Nunc, Roskilde, Denmark). Usually, each culture well contained 10 components in 1 ml of AR. Culture dishes were placed in a shaking incubator (24°C) at 80 oscillation per minute for 6 hour. After culture, medium were saved and kept in a deep freezer (-40°C) until needed for steroid radioimmunoassay.

Steroid radioimmunoassay

P_4, 17α-OHP_4, AD, T and E_2 secreted by the ovarian follicles into the medium during culture were measured by radioimmunoassay (RIA). General assay procedures were adapted from

those described by Fortune (1983) and utilized in previous studies (Kwon *et al.*, 1989, 1991, 1993). Labeled P_4 ([1,2,6,7-^{3}H]-progesterone; 99 Ci/mmol). 17α-OHP_4 ([1,2,6,7-^{3}H]-hydroxy progesterone; 58.5 Ci/mmol), T ([1,2,6,7- ^{3}H]-testosterone, 98 Ci/mmol), and E_2 ([2,4,6,7-^{3}H]-estradiol, 108 Ci/mmol) were obtained from Amersham (Buckinghamshire, England). Labeled AD ([1,2,6,7-^{3}H] -androstenedione, 86.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The antisera of the steroids were produced and evaluated by Dr. Y. D. Yoon (Hanyang University, Seoul). To validate the RIA procedure for measurement of specific steroid in the presence of exogenous precursors, 100 ng/ml of various steroid precursors was added to culture medium (AR) and aliquots (100 μl) were analyzed for specific steroid RIA. Results from such analysis thus provided a mean of assessing the effects of cross-reactivity and served as an additional experimental control. These non-specific binding values were represented in each figure. The P_4 antiserum cross-reacted 14.0% with 5α-dihydroprogesterone, 0.5% with 17α-OHP_4 and 20α-dihydroprogesterone, 0.2% with T, 0.1% with cortisol, and less than 0.01% with other steroids. The 17α-OHP_4 cross-reacted 1.6% with 17α,21-dihydroxyprogesterone, 0.8% with P_4, 0.03% with cortisol, and less than 0.001% with T and E_2. The T antiserum cross-reacted 14.0% with 5α-dihydrotestosterone, 6.0% with 5α-androstenediol, 0.8% with AD, and less than 0.01% with other steroids. The AD antiserum cross-reacted 1.13% with androsterone, 0.5% with T, 0.32% with 5α-dihydrotestosterone, 0.12% with 5α-dihydrotestosterone, and less than 0.01% with other steroids, Each sample was quantified for tritium using a Packard Tri-Carb 1500 liquid scintillation analyzer. Routinely, duplicate steroid standards were included in each assay (P_4, 12.5 - 2000 pg; AD, 2.5 - 500pg). Steroid concentrations were calculated on a microcomputer using SecuRIA software (Packard, Downners Grove, IL). The between- and within-assay coefficients of variation (CVs) for P_4 were 9.2 and 8.5%, respectively. The CVs for 17α-OHP_4 were 8.2 and 6.7%, for T, 9.4 and 7.4%, for E_2, 10.1 and 8.7%, and for AD, 8.4 and 7.2%, respectively. The lower limit of assay sensitivity for P_4 was 12.5 pg, and that for 17α-OHP_4, T, E_2, and AD were 5 pg/follicle.

Statistical analysis

Statistical analysis of data included one or two-way analysis for variance (ANOVA) or student's T-test.

Results

Conversion of P_5 to P_4 by different types of follicular components

Experiments were carried out to examine the conversion of P_5 to P_4 by different types of follicular components *in vitro*. Isolated IFs, GCEOs or THEP layers were cultured for 6 hours in the presence or absence of exogenous P_5 (100 ng/ml) or FPH (0.05 gland/ml). After culture, the amounts of P_4 in medium were measured by RIA. Exogenous P_5 markedly converted to P_4 by GCEOs (2143 pg/follicle) and IFs (2346 pg/follicle) but much less by THEP layers (495 pg/follicle) (Fig. 1)($P<0.01$, when compared with

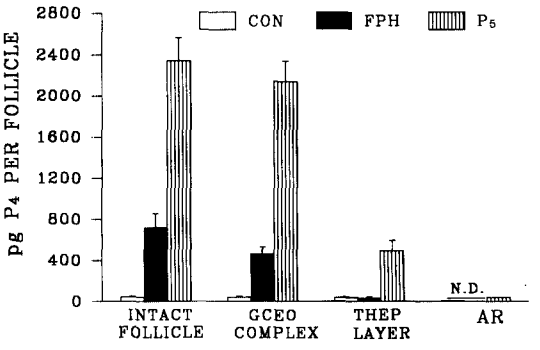

Fig. 1. Conversion of exogenous P_5 to P_4 by different types of follicular components of *R. dybowskii*. Intact follicles (IFs), granulosa cell-enclosed oocytes (GCEOs), and theca/epithelium (THEP) layers were obtained from full-grown follicles and cultured for 6 hr in the presence or absence of P_5 (100 ng/ml) or FPH (0.05 gland/ml). Concentrations of P_4 in the medium were measured by RIA after 6 hr of culture. P_4 levels in AR in the presence of exogenous P_5 or FPH were also measured. Each bar in the figure represents the average number of picogram (mean ± SEM) of P_4 per follicle (n=9, three incubations per animal, 3 animals; n=10, in AR group). N.D., nondetectable.

GCEOs). FPH alone also stimulated P_4 production by GCEOs (436 pg/follicle) and by IFs (725 pg/follicle) but failed to stimulate P_4 production by THEP layers (36 pg/follicle). Without addition of steroid precursors or hormone, very low or nondetectable levels of P_4 were produced by the follicular components examined (<20 pg/follicle). Thus, it is clear that GCEOs are much more efficient than THEP layers in P_4 production ($P<0.01$). Exogenous P_5 also induced a marked increase of P_4 by GCEOs (3350 pg/follicle) and IFs (3477 pg/follicle), but less increase by THEP layers (672 pg/follicle) and least increase by P-THEP layers (134 pg/follicle)(Fig. 2). Thus, P-THEP layer was much less efficient than THEP in converting P_5 to P_4 ($P<0.01$). The exogenous P_5 (100 ng/ml) detected as 60 pg/follicle of P_4 because of cross reactivity (Fig. 2).

Conversion of P_4 to 17α-OHP_4 by different types of follicular components

Conversion of P_4 to 17α-OHP_4 by different types of follicular components were examined. Exogenous P_4 markedly increased 17α-OHP_4 levels by GCEOs (1118 pg/follicle) and IFs (1333 pg/follicle) but much less by THEP layers (289 pg/follicle) (Fig. 3)($P<0.01$, when compared with GCEOs). Addition of P_5 also increased 17α-OHP_4 levels markedly by GCEOs (668 pg/follicle) and IFs (1183 pg/follicle) but not by THEP layers (62 pg/follicle). Likewise, FPH alone also stimulated 17α-OHP_4 production by GCEOs (394 pg/follicle) and by IFs (623 pg/follicle) but failed to stimulate 17α-OHP_4 production by THEP layers (43 pg/follicle). Without addition of steroid precursors or hormone, very low or nondetectable levels of P_4 were produced by the follicular components examined (<20 pg/follicle). Thus, it is clear that GCEOs were much more efficient than THEP layers in 17α-OHP_4 production ($P<0.01$). Exogenous P_4 resulted in a marked increase in 17α-OHP_4 by GCEOs (844 pg/follicle) and IFs (901 pg/follicle), but much less by THEP layers (310 pg/follicle) and by P-THEP layers (283 pg/follicle). As 17α-OHP_4 levels in AR was detected as 261 pg/follicle equivalent in the presence of exogenous P_4 (100 ng/ml) because of cross reactivity of 17α-OHP_4 antibody with P_4, the real amounts of 17α-OHP_4 produced by P-THEP seems to be negligible (Fig. 3).

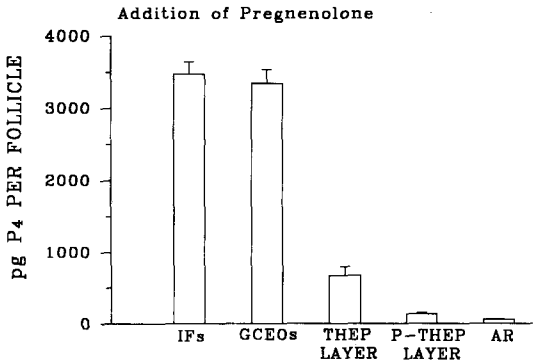

Fig. 2. Conversion of exogenous P_5 to P_4 by different types of THEP layers. Granulosa cell-enclosed oocytes (GCEOs), theca/epithelium (THEP) layers, P-THEP layers (granulosa cells free THEP layer) were obtained from full-grown follicles and cultured for 6 hr in the presence of P_5 (100 ng/ml). Concentrations of P_4 in the medium were measured by RIA after 6 hr of culture. Each bar in the figure represents the average number of picogram (mean ± SEM) of P_4 per follicle (n=9, three incubations per animal, 3 animals; n=10, in AR group). N.D., nondetectable.

Conversion of 17α-OHP_4 to AD by different types of follicular components

Exogenously added 17α-OHP_4 markedly increased levels of AD by GCEOs (1415 pg/follicle) and IFs (561 pg/follicle) but not by THEP layers (nondetectable)(Fig. 4)($P<0.01$, when compared with GCEOs). Considerable levels of AD were also produced by GCEOs and IFs in the presence of P_5 or P_4 (138 - 497 pg/follicle) but not by THEP layers (nondetectable). FPH alone also stimulated AD production by GCEOs (394 pg/follicle) and by IFs (623 pg/follicle) but not by THEP layers (nondetectable). Thus, it is clear that GCEOs were much more efficient than THEP layers in AD production ($P<0.01$).

Conversion of AD to T by different types of follicular components

Conversion of AD to T by different types of follicular components were examined. Exogenously added AD markedly increased the levels of T by THEP layers (2594 pg/follicle) and

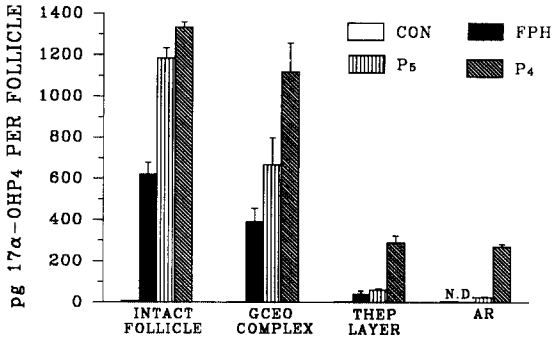

Fig. 3. Conversion of exogenous P_4 to 17α-OHP_4 by different types of follicular components of *R. dybowskii*. Intact follicles (IFs), granulosa cell-enclosed oocytes (GCEOs), and theca/epithelium (THEP) layers were obtained from full-grown follicles and cultured for 6 hr in the presence or absence of P_4 or P_5 (100 ng/ml) or FPH (0.05 gland/ml). Concentrations of 17α-OHP_4 in the medium were measured by RIA after 6 hr of culture. Seventeen alpha hydroxyprogesterone levels in AR in the presence of exogenous P_4, P_5 or FPH were measured. Each bar in the figure represents the average number of picogram (mean ± SEM) of 17α-OHP_4 per follicle (n=9, three incubations per animal, 3 animals; n=10, in AR group). N.D., nondetectable.

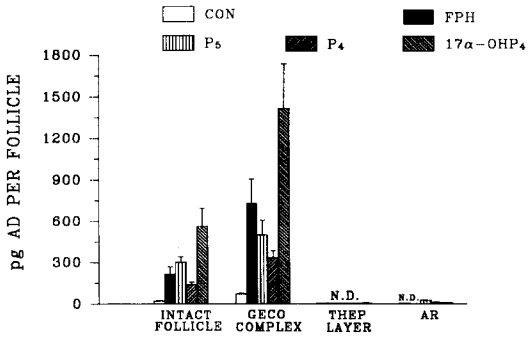

Fig. 4. Conversion of exogenous precursors to AD by different types of follicular components of *R. dybowskii*. Intact follicles (IFs), granulosa cell-enclosed oocytes (GCEOs), and theca/epithelium (THEP) layers were obtained from full-grown follicles and cultured for 6 hr in the presence or absence of 17α-OHP_4 or other precursors (P_5 or P_4; 100 ng/ml, each) or FPH (0.05 gland/ml). Concentrations of AD in the medium were measured by RIA after 6 hr of culture. AD levels in AR in the presence of exogenous P_5, P_4, 17α-OHP_4 or FPH were also measured. Each bar in the figure represents the average number of picogram (mean ± SEM) of AD per follicle (n=9, three incubations per animal, 3 animals; n=10, in AR group).

IFs (2223 pg/follicle) but much less by GCEOs (339 pg/follicle)(Fig. 5)($P<0.01$, when compared with THEP layers). Considerable amounts of T were also produced by IFs in the presence of P_5, P_4 or 17α-OHP_4 (630 - 1692 pg/follicle) but very low or nondetectable levels of T were produced by THEP layers or GCEOs (25 - 100 pg/follicle). FPH alone stimulated T production by IFs (963 pg/follicle) but failed to stimulate T production by THEP layers (nondetectable) or GCEOs (65 pg/follicle). Thus, it is clear that THEP layers were much more efficient than GCEOs in converting exogenous AD to T, but the tissue could not produce T alone in response to FPH (Fig. 5).

Conversion of T to E_2 by different types of follicular components

Conversion of T to E_2 by different types of follicular components were also examined. Exogenous addition of T markedly increased the levels of E_2 by GCEOs (551 pg/follicle) and IFs (887 pg/follicle) but not by THEP (<20 pg/follicle)(Fig. 6)($P<0.01$, when compared with GCEOs). Likewise, addition of other steroid precursors (P_5, P_4, 17α-OHP_4 or AD) or FPH significantly increased E_2 secretion by GCEOs (173 - 226 pg/follicle) and IFs (331 - 627 pg/follicle) but not by THEP layers. Without addition of steroid precursors or hormone, low levels or nondetectable levels of E_2 were produced by the follicular components examined. Thus, it is evident that GCEOs were more efficient than THEP layers in producing E_2 ($P<0.01$).

Discussion

The data presented here demonstrated that granulosa cells are main sites for production of P_4, 17α-OHP_4, AD and E_2 whereas theca layers are responsible for synthesis of T in ovarian follicles of *R. dybowskii*. Thus, it is evident that bidirectional cooperation of both two types of cells is essential for production of T and E_2 in amphibian ovarian follicles. Further, the data suggested that the conversion of P_5 to 17α-OHP_4 by theca layer is negligible when compared with that by granulosa cells. Taken together, These data indicate that the

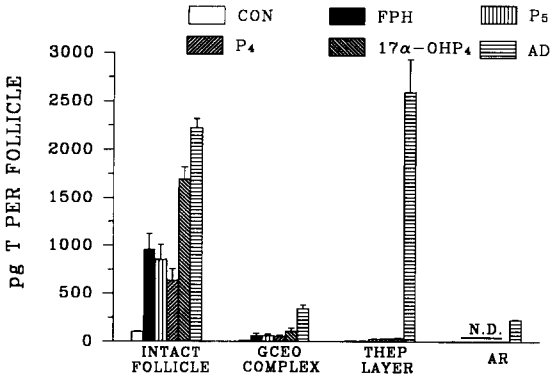

Fig. 5. Conversion of exogenous AD to T by different types of follicular components of *R. dybowskii*. Intact follicles (IFs), granulosa cell-enclosed oocytes (GCEOs), and theca/epithelium (THEP) layers were obtained from full-grown follicles and cultured for 6 hr in the presence or absence of AD or other precursors(P_5, P_4 or 17α-OHP_4; 100 ng/ml, each) or FPH (0.05 gland/ml). Concentrations of T in the medium were measured by RIA after 6 hr of culture. T levels in AR in the presence of exogenous P_5, P_4, 17α-OHP_4, AD or FPH were measured. Each bar in the figure represents the average number of picogram (mean ± SEM) of T per follicle (n=9, three incubations per animal, 3 animals; n=10, in AR group).

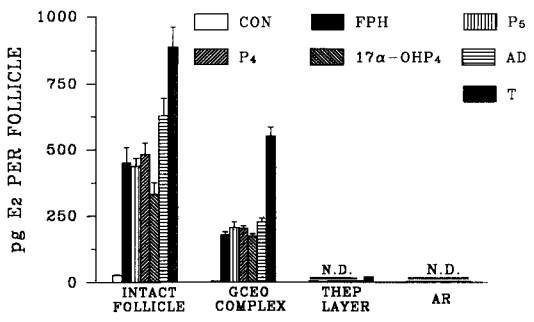

Fig. 6. Conversion of exogenous T to E_2 by different types of follicular components of *R. dybowskii*. Intact follicles (IFs), granulosa cell-enclosed oocytes (GCEOs), and theca/epithelium (THEP) layers were obtained from medium-sized follicles and cultured for 6 hr in the presence or absence of T or other precursors (P_5, P_4, 17α-OHP_4, AD; 100 ng/ml, each) or FPH (0.05 gland/ml). Concentrations of E_2 in the medium were measured by RIA after 6 hr of culture. E_2 levels in AR in the presence of exogenous P_5, P_4, 17α-OHP_4, AD, T or FPH were also measured. Each bar in the figure represents the average number of picogram (mean ± SEM) of E_2 per follicle (n=9, three incubations per animal, 3 animals; n=10, in AR group).

two-cell type model for follicular steroidogenesis in amphibians, which based on the data from *R. nigromaculata*, is applicable to *R. dybowskii* and that there are no minor pathway from P_5 to 17α-OHP_4 in the theca layer, which was proposed in previous report (Kwon and Ahn, 1994).

Since higher levels of P_4 or 17α-OHP_4 were produced by GCEOs than by THEP layers in the presence of precursors (P_5 or P_4) or FPH (Figs. 1 and 3), it is evident that activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase are higher in the GCEOs than in THEP layers. Moreover, granulosa cell free THEP (P-THEP) layers produced much lower levels of P_4 or 17α-OHP_4 than that of THEP layer (Figs. 2 and 3)($P<0.01$). These results suggest that the conversion of P_5 to 17α-OHP_4 by THEP layers was due to the granulosa cells remained in THEP layers during isolation procedure rather than to the THEP layer. Efficient production of AD or E_2 by GCEOs (Figs. 4 and 6) also suggest that activities of of $C_{17,20}$-lyase and aromatase are higher in granulosa cells than in theca layer.

In contrast, much higher concentrations of T were produced by THEP layers than by GCEOs in the presence of AD (Fig. 5), suggesting that the activity of 17β-hydroxysteroid dehydrogenase (17β-HSD) is higher in theca layer than granulosa cells. If we accept the fact that 17β-HSD activity is higher in the theca layer, it is resonable that higher levels of AD was produced by GCEOs than by IFs in the presence of precursors (P_5, P_4 or 17α-OHP_4) because GCEOs are deficient in 17β-HSD activity for conversion of AD to T (Fig. 4). This fact suggests that AD will be accumulated in GCEOs, but will be converted to other steroids (T and E_2) by IFs.

From the fact of that P_4, 17α-OHP_4 and AD produced by GCEOs are in similar levels to those produced by intact follicles (IFs) in response to FPH (Figs. 1-4), these steroids seem to be produced solely by granulosa cells. Interestingly, however, GCEOs produced lower levels of E_2 than IFs in the presence of precursors or FPH (Fig. 6)($P<0.01$). This fact suggests that cooperation between the two types of cells is required for

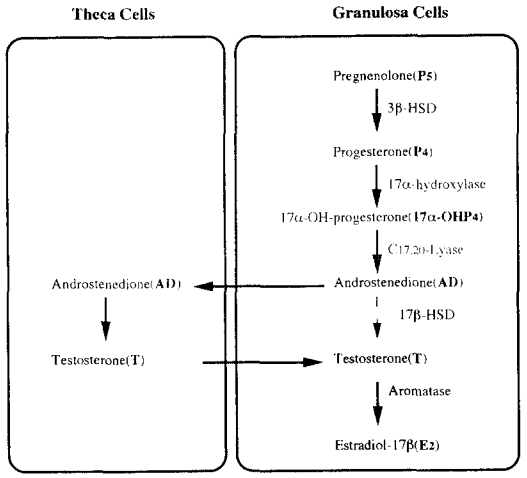

Fig. 7. A modified two-cell type model for follicular steroidogenesis in *Ranas*. Granulosa cells are main site for progesterone, 17α-hydroxyprogesterone, androstenedione and estradiol, whereas theca cells are that for testosterone. Bidirectional cooperations are require for efficient production of T and E_2.

efficient production of these steroid. On the basis of present data, we propose a modified two-cell type model depicted in Figure 7. This two-cell type model is basically the same as the previous model proposed with *R. nigromaculata*. However, we make it clear that conversion of P_5 to 17α-OHP_4 do not occur in theca layer in this model.

Interestingly, the steroidogenic pathway in *Rana* ovarian follicles is very similar to that observed in some teleost fishes and mammals. Theca layers contribute to E_2 production by synthesizing androgens (AD and T) which are aromatized by granulosa cells to E_2 in fishes (Kagawa *et al.*, 1982; Nagahama and Adachi, 1985) and in mammals (reviewed by Gore-Langton and Armstrong, 1988). However, it appeared that only one step of biosynthetic pathway is present in follicles of *Rana* while additional steps are present in fish and mammals. However, in some fishes theca cell was known to play a minor role in follicular steroidogenesis. For example, Petrino *et al.*, (1991) demonstrated that granulosa cells produced various steroids including E_2 without aid of theca cells in *Fundulus heteroclitus*. In contrast, granulosa cells were found to produce progestins, which are converted to T by theca cells and eventually to E_2 by these cells in hens (Huang *et al.*, 1979; Bahr *et al.*, 1983). Other investigators found that roles of theca externa and interna cells are different and proposed three- or multiple cell type models for steroidogenesis in avian follicles (Poter *et al.*, 1989; Nitta *et al.*, 1991). According to the multiple cell type model, theca interna produce progestins and androgens, and theca externa produce androgens and estrogens. Thus, roles of granulosa cells and theca cells in avian follicles are more complex than those of *Rana*.

In summary, present studies demonstrated that granulosa cells produced P_4, 17α-OHP_4, AD and E_2, whereas theca layers produced T in *R. dybowskii* ovarian follicles *in vitro*. This fact suggests that cooperation of two types of cells are required for efficient production of T and E_2. Thus, the two-cell type model for follicular steroidogenesis in amphibians previously proposed in our laboratory is applicable to other species of *Rana*. Further, this study suggests that a minor pathway ($P_5 \rightarrow$ 17α-OHP_4) in theca, which was proposed in previous report, is not present in *Rana* ovarian follicles.

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북방산개구리 여포의 스테로이드생성과정에 협막세포와 난구세포의 역할

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본인 등은 참개구리를 이용하여 여포의 스테로이드 생성에 관한 two-cell type model을 제시한 바 있다. 본 연구에서는 이 model이 북방산개구리(*R. dybowskii*)에도 적용되는지와 협막세포층에 minor pathway($P_5 \rightarrow 17\alpha\text{-}OHP_4$)가 있는지의 여부를 조사하였다. 이를 위하여 북방산개구리 난소로부터 intact follicles(IFs), granulosa cell-enclosed oocytes(GCEOs), theca/epithelium(THEP) layers 및 난구세포가 포함되어 있지 않은 순수한 theca/epithelium(P-THEP) layer를 미세해부기술로 분리해 내었다. 이들 여포조직들을 전구 스테로이드들이나 개구리 뇌하수체추출물(FPH)이 포함되어 있는 배양액에서 6시간 배양한 후, 각 여포조직에 의해 전환된 산물스테로이드의 양을 방사면역측정법으로 조사하였다. 외부에서 첨가된 P_5와 P_4는 GCEOs와 IFs에 의하여 효율적으로 P_4 혹은 $17\alpha\text{-}OHP_4$로 전환되었으나 THEP에 의해서는 조금밖에 전환되지 않았다. 더욱이 순수한 협막층(P-THEP)에 의해서는 전환이 거의 이루어지지 않았다. $17\alpha\text{-}OHP_4$ 및 testosterone 역시 GCEOs와 IFs에 의해서 estradiol(E_2) 및 androstenedione(AD)으로 각각 전환되었으나 THEP에 의해서는 전환되지 않았다. 반면에, AD는 THEP과 IFs에 이해서만 T로 전환되어졌으며, AD를 제외한 다른 전구스테로이드들은 THEP 의해서도 T로 전환 되지못했다. 이러한 결과들은 P_4, $17\alpha\text{-}OHP_4$, AD 및 E_2는 주로 난구세포에서 생성되고, T는 주로 협막세포에서 생성되며 T나 E_2의 효율적인 생성에 이들 두 세포의 협조가 필요하다는 것을 말해준다. 이는 참개구리에서 제시한 two-cell type model이 북방산개구리 여포의 스테로이드 생성과정에도 적용되며 협막세포층에는 minor pathway($P_5 \rightarrow 17\alpha\text{-}OHP_4$)가 존재하지 않음을 또한 보여주고 있다.