Effects of Organotin Compounds on Follicular Steroidogenesis in Frogs

Hyuk Bang Kwon[1], Seung Chang Kim[1], Anna Kim[1], Sung Ho Lee[2] and Ryun Sup Ahn[3†]

[1]Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, GwangJu 500-757, Korea

[2]Dept. of Life Science, Sangmyung University, Seoul 110-743, Korea

[3]Graduate School of Complementary and Alternative Medicine, CHA Medical University, Seoul 135-913, Korea

ABSTRACT : Some organotin compounds such as butyltins and phenyltins are known to induce impo-sex in various marine animals and are considered to be endocrine disruptors. In this study, the effect of organotins on follicular steroidogenesis in amphibians was examined using ovarian follicles of *Rana dybowskii* and *Rana catesbeiana*. Isolated follicles were cultured for 6 or 18 h in the presence and absence of frog pituitary homogenate (FPH) or various steroid precursors, and the levels of product steroids in the culture media oassay. Among the butyltin compounds, tributyltin (TBT) strongly and dose-dependently inhibited the FPH-induced synthesis of pregnenolone (P_5) and progesterone (P_4) by the follicles. TBT also strongly suppressed the conversion of cholesterol to P_5 and partially suppressed the conversion of P_5 to P_4. A high concentration of dibutyltin (DBT) also inhibited steroidogenesis by the follicles while monobutyltin and tetrabutyltin had negligible effects. The toxic effect of TBT or DBT was irreversible and a short time of exposure (30 min) was enough to suppress steroidogenesis. All the phenyltin compounds significantly inhibited FPH-induced P_5 synthesis by the follicles. The effective dose of 50% inhibition by diphenyltin was 0.04 μM and those of monophenyltin and triphenyltin were 0.24 μM and 0.3 μM, respectively. However, none of the phenyltin compounds significantly suppressed the conversion of P_4 to 17α-hydroxyprogesterone (17α-OHP) (by 17α-hydroxylase), 17α-OHP to androstenedione (AD) (by C_{17-20} lyase), or AD to testosterone by the follicles. Taken together, the data show that among the steroidogenic enzymes, P450scc in the follicles is the most sensitive to organotin compounds and that an amphibian follicle culture system can be a useful screening model for endocrine disruptors.

Key words : Amphibian, Endocrine disruptors, Steroidogenesis, Organotin compounds.

INTRODUCTION

The use of tributyltin (TBT) in antifouling paints and wood preservatives for ships is responsible for world-wide contamination of fresh water and marine environments. It is well known that these compounds, when released into the environment, can cause significant declines in the populations of marine oyster species (Laughlin & Linden, 1987). In particular, TBT is known to be highly toxic toward various non-target marine organisms (Fent, 1996). Phenyltin compounds are widely used in agriculture as fungicides and in consumer products (Kannan & Lee, 1996). They contaminate the environment and have toxic effects on aquatic and terrestrial animals including humans (Kimbrough, 1976; Kannan et al., 1997; Maguire, 2000; Nielsen & Strand, 2002). In particular, some studies have shown that they are responsible for the presence of imposex in a population of gastropods (Horiguchi et al., 1998; Pellizzato et al., 2004). Also, derivatives of these compounds are known to cause development and reproductive toxicity in

† Corresponding author: Ryun Sup Ahn, Ph.D., Graduate School of Complementary and Alternative Medicine, CHA Medical University, Yuksam-dong 605, Kangnam, Seoul 135-080, Korea. Tel: +82-2-3468-3323, Fax: +82-2-514-9084, E-mail: ryunsup@yahoo.co.kr

mammals by disrupting the endocrine system. Recently, considerable amounts of TBT and triphenyltin (TPT) were found to have accumulated in starfish and bivalves collected from the coastal area of the Korean peninsula (Shim et al., 2005). Many reports have also indicated that organotins suppress various steroidogenic enzymes in different animal tissues. For example, TBT was found to inhibit the activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase, but not that of 17β-hydroxysteroid dehydrogenase (17β-HSD) in rat testes microsomes (McVey & Cooke, 2003). The toxic effects of butyltin compounds on steroidogenic enzymes have also been examined in cows (Schoenfelder et al., 2003; Yamazaki et al., 2005), pigs (Ohno et al., 2005) and humans (Cooke, 2002). Our previous studies also have shown that some butyltins suppressed follicular steroidogenesis in the frog; *R. catesbeiana* (Ahn et al., 2007).

The aim of this research is to determine whether phenyltins also suppress follicular steroidogenesis in amphibians and to compare the responsiveness of follicles obtained from two different species of frogs to organotins. The present data showed that phenyltins like butyltins are very effective in suppressing follicular steroidogenesis in amphibians and that frog follicles obtained from two different species of frogs exhibit similar responses to organotins. This finding supports the idea that an amphibian follicle culture system can be a useful screening model for organic endocrine disruptors.

MATERIALS AND METHODS

1. Animals

Mountain frogs (*Rana dybowskii*) were collected in October-December from streams in the Chonnam area, in the southwestern part of the Korean peninsula, and bullfrogs (*R. catesbeiana*) were collected from fields in the Chonbuk area, in the western part of the Korean peninsula. The animals were kept in stainless steel boxes containing water and stones to simulate their natural habitat. They were acclimatized to these conditions for several days and after one week they were used for experimentation.

2. Hormones and Reagents

Steroids (25-OH-cholesterol, pregnenolone, P_5; progesterone, P_4) were purchased from Sigma-Aldrich (St. Louis, Mo). All the organotin compounds, monobutyltin (MBT), dibutyltin (DBT), TBT, tetrabutyltin (TeBT), monophenyltin (MPT), diphenyltin (DPT), and triphenyltin (TPT) were also purchased from Sigma-Aldrich (St. Louis, Mo). Steroid precursors were dissolved in a vehicle composed of ethanol and propylene glycol (1:1). The final concentration of the vehicle in the medium was below 0.5% v/v and was found not to influence steroid secretion (data not shown). All organotins were dissolved in dimethylsulfoxide (DMSO). Different concentrations of reagents were prepared by dilution of the stock solution with DMSO. Frog pituitary homogenate (FPH) was prepared as previously described (Kwon et al., 1993; Kwon & Ahn, 1994) and used to stimulate follicular steroidogenesis.

3. Follicle Culture

After animals were killed by decapitation, ovaries were removed immediately and divided into fragments in amphibian Ringer's solution (AR). Follicles were manually isolated under magnification from other ovarian tissue using watchmaker's forceps under a dissecting microscope. Isolated follicles from *R. dybowskii* were cultured for 6 h and those from *R. catesbeiana* for 16 h AR in the presence or absence of FPH (0.1 pituitary/2 ㎖) and/or with various exogenous substrates. *In vitro* culture was carried out at room temperature (23-25℃, with 10 follicles in 1 ㎖ of AR per well [24 wells per dish; Nunc, Denmark]). The duration of follicle culture and the dose of FPH were chosen on the basis of our previous data from *Rana* (Kwon & Schuetz, 1985; Kwon et al., 1991). Control and experimental groups were cultured in triplicate using follicles from each animal and each experiment was replicated with

follicles from 3-6 frogs.

4. Follicle Extraction and Sample Preparation for RIA

As P_5 was not secreted into the culture medium, P_5 was extracted from follicles by a procedure previously described by Kwon and Schuetz (1985). After culture, follicles were extracted in the culture wells using methanol (GR, Merck, 1 ㎖ methanol per 10 follicles per well) and shaken for 15 min (100 oscillations per min). Methanol extracts were dried under vacuum using a centrifugal lyophilizer (Savent SVC-100; Hickville, USA), and reconstituted using 0.05 M gelatin phosphate buffered saline (GPBS, pH 7.2). Extracts in GPBS were assayed without further purification. For other steroids, the culture medium was used directly for measuring the steroids by RIA.

5. Steroid Radioimmunoassay

Steroids secreted by the ovarian follicle into the medium and those present in the follicle extracts, were measured by radioimmunoassay (RIA). The general assay procedure was adapted from those described in previous studies (Kwon et al., 1993; Kwon & Ahn, 1994). Labeled P_5 ([7-^{3}H (N)]-pregnenolone; 25 Ci/mmol) was purchased from PerkinElmer Life Science (Boston, MA, USA). Labeled P_4 (1,2,6,7-^{3}H-progesterone; 99 Ci/mmol), 17α-OHP ([1,2,6,7,-^{3}H])-hydroxyprogesterone; 58.5 Ci/mmol), labeled AD ([1,2,6,7-^{3}H]-androstenedione; 86.1 Ci/mmol) and T ([1,2,6,7-^{3}H]-testosterone; 98 Ci/mmol) were obtained from Amersham (Buckinghamshire, England). The steroid antiserum against AD was purchased from Sigma (St. Louis, MO, USA) and those against P_5, P_4, 17α-OHP, and testosterone (T) were obtained from Biogenesis (England). The P_5 antiserum cross-reacted 19% with P_4, and less than 3% with 17α-OHP, AD, or T. The P_4 antiserum cross-reacted 4% with T, 2% with 17α-OHP and less than 0.1% with other steroids. The 17α-OHP antiserum cross-reacted 0.25% with P_4, and less than 0.1% with other steroids. The AD antiserum cross-reacted 4.5% with T, and less than 0.1% with other steroids. The T antiserum cross-reacted 3.3% with 11β-hydroxy testosterone, and less than 0.1% with E_2, P_4 or other steroids. Each sample was quantified for tritium using a Packard Tri-Carb 2900TR liquid scintillation analyzer. Routinely, duplicate steroid standards were included in each assay (5-2,000 pg). The between and within assay coefficients of variation (CV) for P_5 were 9.2% and 9.3%, respectively. The CVs for P_4 were 11.5% and 6.7%, 6.2% and 6.5% for 17α-OHP, 5.6% and 6.9% for AD, 6.3% and 7.9% for T, respectively.

6. Statistical Analysis

Data (% of control) for all experiments were transformed using an arcsine-square root transformation and were analyzed by one-way analysis of variance (ANOVA) or Student's *t*-test using Prism statistical software (Kwon et al., 1993; Kwon & Ahn, 1994). Differences were considered to be significant when their probability was less than 0.05 or 0.01.

RESULTS

1. Effect of Butyltins on P_5 and P_4 Synthesis by Ovarian Follicles of *R. dybowskii In Vitro*

In previous studies, we showed that butyltin compounds suppress follicular steroidogenesis of *R. catesbeiana* (Ahn et al., 2007). In the present experiments, we performed similar experiment with the ovarian follicles of the Korean frog, *R. dybowskii* in order to know whether the follicles from the two species respond similarly to butyltin compounds. The isolated follicles were incubated for 6 h in AR in the presence of FPH and/or various concentrations of butyltins (0.01-100 μM) and P_5 in follicles or P_4 in culture media were measured by RIA. Fig. 1 shows that TBT strongly suppressed FPH-induced P_5 and P_4 synthesis by the follicles in a dose-dependent manner (ED_{50}, 0.7 μM) (Fig. 1). DBT also inhibited P_5 and P_4 synthesis in a similar way but MBT and TeBT were much less inhibitory (Fig. 1).

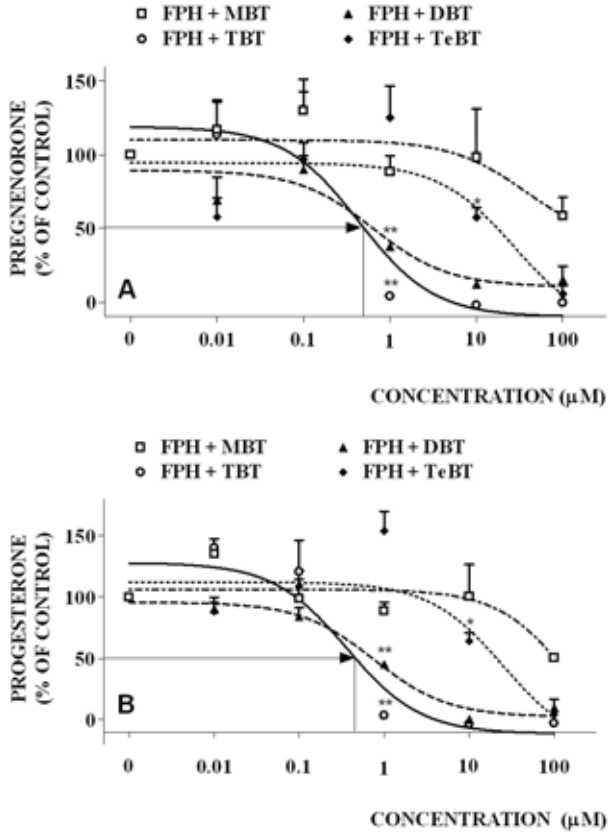

Fig. 1. Effects of butyltins on FPH-induced P_5 and P_4 synthesis by ovarian follicles of *Rana dybowskii*. Isolated follicles were cultured for 6 h in the presence of FPH (0.05 gland/㎖) with or without various concentrations of butyltins. Each point in the figure represents relative P_5 (A) or P_4 (B) levels (mean ± SEM, % of control) (n=6, duplicate incubation, 3 animals). * $p<0.05$, ** $p<0.01$ when compared to control by *t*-test.

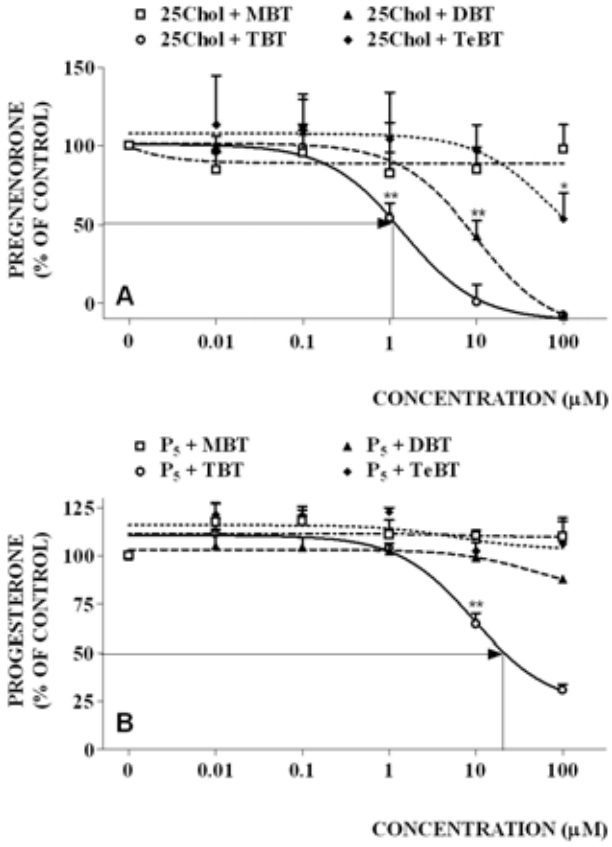

Fig. 2. Effects of butyltins on the conversion of 25-OH-cholesterol to P_5 and P_5 to P_4 by *R. dybowskii* follicles. Isolated follicles were cultured for 6 h in the presence of 25-OH-Cholesterol (100 ng) or P_5 (100 ng) with or without various concentration of butyltin. Each point in panel A represents relative P_5 levels (mean ± SEM, % of control) and panel B represents relative P_4 levels (n=6 duplicate incubations, 3 animals).

2. Effect of Butyltins on the Conversion of 25-OH-Cholesterol to P_5 and P_5 to P_4 by Ovarian Follicles of *R. dybowskii*

The effect of butyltin compounds on the conversion of cholesterol to P_5 by P450scc was examined. As it was known that exogenous cholesterol could not be utilized by the follicles, the steroid precursor 25-OH-cholesterol (25 Chol) was used as precursor for P_5 synthesis. Isolated follicles were cultured for 6 h in the presence of 25-OH-cholesterol (100 ng/㎖) with or without various concentrations of butyltins; after culture, P_5 levels in the follicles were measured. Fig. 2 shows that TBT strongly suppressed the conversion of 25-OH-cholesterol to P_5 in a dose-dependent manner (ED_{50}, 1 μM). DBT also suppressed the conversion in high doses (>10 μM), but MBT and TeBT failed to suppress the conversion (Fig. 2A). The effect of butyltins on the conversion of P_5 to P_4 by the follicles was also examined (Fig. 2B). Interestingly, only TBT suppressed the conversion of P_5 to P_4 at relatively high doses (ED_{50}, 13 μM), and other butyltins failed to

suppress the conversion even at the highest doses of butyltins (100 μM). Thus it is evident that TBT and DBT suppressed the enzyme P450scc enzyme that is responsible for the conversion of cholesterol to P_5 and were much less effective inhibitors of the enzyme 3β-HSD that is responsible for the conversion of P_5 to P_4.

3. Irreversible Inhibition by TBT and DBT of Follicular Steroidogenesis in *R. dybowskii*

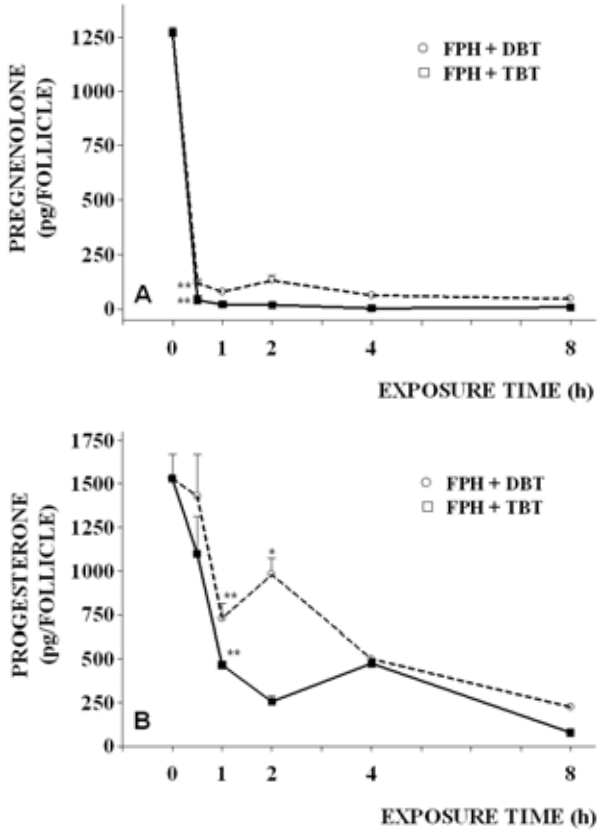

Fig. 3. Irreversible effects of butyltins on FPH-induced P_5 or P_4 production by *R. dybowskii* follicles. Isolated ovarian follicles were exposed to 10 μM butyltins (DBT and TBT) for designated time periods (0.5, 1, 2, 4 and 8 hours). After washing, the follicles were cultured further for 18 h in the presence of FPH. After culture, P_5 in the follicles (panel A) or P_4 in the medium (panel B) were measured. Each point in the figure represents relative P_5 or P_4 levels (mean ± SEM, % of control) (n=6, duplicate incubations, 3 animals).

In order to know whether the toxic effect of the butyltins is reversible, we exposed isolated follicles to high doses of TBT or DBT (10 μM) for various periods (0.5-8 h) and then transferred them to plain medium for further culture in the presence of FPH for 6 h. After culture, P_5 in the follicles and P_4 in the medium were measured. A half-hour exposure to the butyltins completely suppressed FPH-stimulated P_5 synthesis by the follicles (Fig. 3A). P_4 production by the follicles was also suppressed when they are exposed for 1 h or longer (Fig. 3B). Thus it is evident that the toxic effect of butyltins is irreversible.

4. Effects of Phenyltins on FPH-induced Pregnenolone (P_5) Synthesis by the Ovarian Follicles of *R. catesbeiana*

The effect of phenyltins on the FPH-stimulated P_5 synthesis by the follicles was examined. The activity of P450scc can be estimated from the amount of P_5 produced by the follicles in response to phenyltins. Isolated follicles were cultured for 16 h in the presence of FPH (0.05 gland/mℓ) and/or phenyltin compounds of various concentrations (0.01-100 μM). After culture, the amount of P_5 in the follicles was measured by RIA. A considerable amount of P_5 was

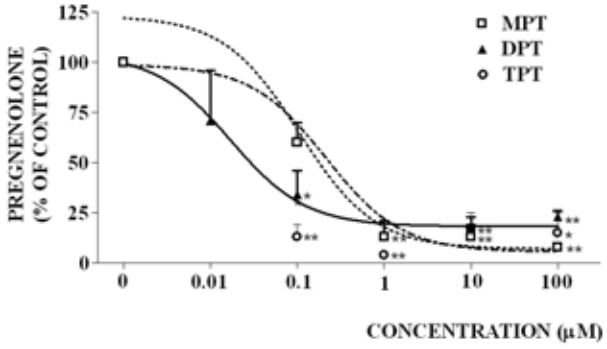

Fig. 4. Effects of phenyltins on FPH-induced P_5 production by *R. catesbeiana* follicles *in vitro*. Isolated follicles were cultured for 18 h in the presence of FPH (0.05 gland/mℓ) with or without various concentrations of phenyltins. Each point in the figure represents relative P_5 levels (mean ± SEM, % of control) (n=6, duplicate incubations, 3 animals). * $p<0.05$, ** $p<0.01$ when compared to control by *t*-test.

produced in response to FPH by the follicles (658-1,058 pg/follicle, n=6). As shown in Fig. 4, all the phenyltins strongly suppressed FPH-induced P_5 production by the follicles in a dose-dependent manner (Fig. 4). DPT was most effective in the inhibition (ED_{50}, 0.04 μM) and almost completely suppressed P_5 synthesis from very low concentration (0.01 μM). MPT and TPT also inhibited the steroid synthesis significantly from 0.1 μM (ED_{50}, 0.24 μM and 0.3 μM, respectively). Therefore, the toxicities of phenyltin compounds for P450scc were in the order DPT > MPT = TPT.

5. Effects of Phenyltins on the Conversion of P_5 to P_4 or P_4 to 17α-OHP by Ovarian Follicles of *R. catesbeiana*

To assess the effect of phenyltin compounds on 3β-hydroxysteroid dehydrogenase (3β-HSD), which converts P_5 to P_4, or 17α-hydroxylase, which converts P_4 to 17α- OHP, in the follicles, we cultured ovarian follicles for 16 h in medium containing P_5 (100 ng/mℓ) or P_4 (100 ng/mℓ) ; after culture, the amount of P_4 converted from P_5 or 17α- OHP converted from P_4 was measured in the medium. As shown in Fig. 5A, all the phenyltins seemed to suppress the conversion of P_5 to P_4 at high doses (>10 μM). However, the P_4 levels converted from P_5 exhibited considerable variation and were not significantly suppressed by the phenyltins in the medium (Fig. 5A). Thus it seems that the phenyltins partially, but not significantly, suppressed 3β-HSD activity in the follicles. Interestingly, the conversion of P_4 to 17α-OHP by follicles was not affected by phenyltins in the medium even at the highest dose examined (100 μM) (Fig. 5B). TPT instead slightly, but not significantly, increased the accumulation of 17α-OHP. The accumulation of 17α-OHP likely resulted from the inhibition of its metabolism rather than from stimulation of 17α-hydroxylase by the TPT (Fig. 5B). These results indicated that 17α-hydroxylase activity is not sensitive to phenyltin compounds.

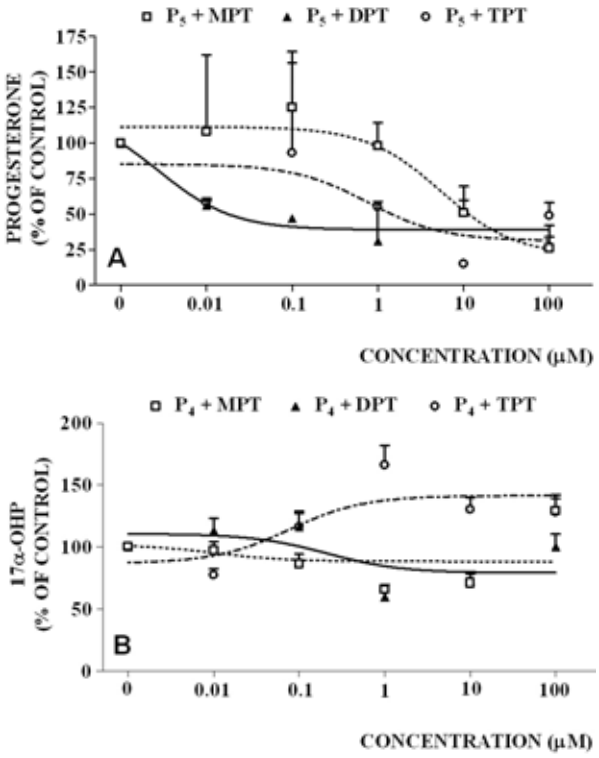

Fig. 5. Effects of phenyltins on the conversion of P_5 to P_4 or P_4 to 17α-OHP by ovarian follicles of *R. catesbeiana*. Isolated follicles were cultured for 18 h in the presence of P_5 or P_4 with or without various concentrations of phenyltins. After culture, P_4 or 17α-OHP in medium were measured by RIA. Each point in panel A represents relative P_4 levels and panel B represents relative 17α-OHP levels (mean ± SEM, % of control) (n=6, duplicate incubations, 3 animals).

6. Effects of Phenyltin Compounds on the Conversion of 17α-OHP to AD and AD to T by the Ovarian Follicles of *R. catesbeiana*

To ascertain whether phenyltins have any effect on the activity of $C_{17\text{-}20}$ lyase which converts 17α-OHP into androstenedione (AD) or 17β-hydroxysteroid dehydrogenase (17β-HSD) which converts AD to T in the follicles, we cultured ovarian follicles in medium containing 17α-OHP (100 ng/mℓ) or AD as precursors, and measured levels of product steroids (AD and T, respectively) in the medium were measured after 16 h of culture. The amount of AD

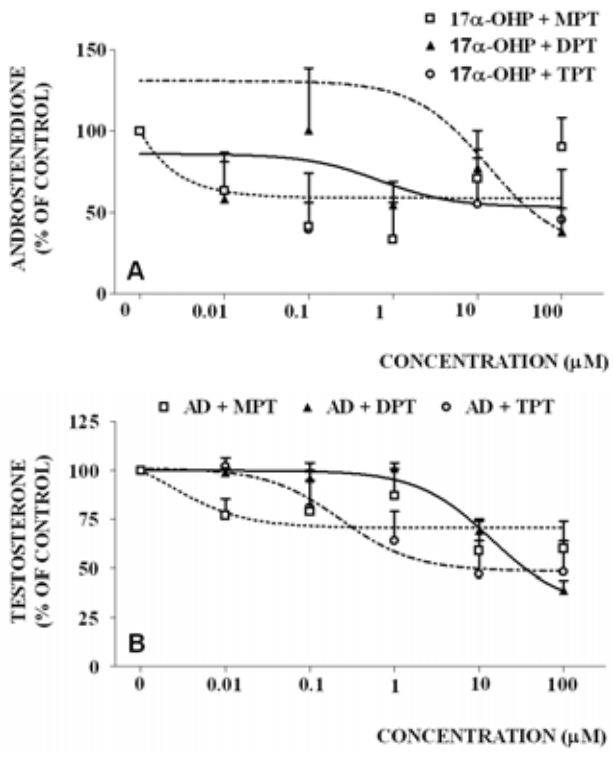

Fig. 6. Effects of phenyltins on the conversion of 17α-OHP to AD or AD to T by ovarian follicles of *R. catesbeiana in vitro*. Isolated follicles were cultured for 18 h in the presence of 17α-OHP or AD with or without various concentrations of phenyltins. After culture, AD or T in the medium were measured by RIA. Each point in panel A represents relative AD levels and panel B represents relative T levels (mean ± SEM, % of control) (n=6, duplicate incubations, 3 animals).

secreted by the control follicles was in the range of 11-235 ng/follicle (n=6). Without addition of 17α-OHP, barely detectable levels of AD were produced. As shown in Fig. 6A, all the phenyltins (MPT, DPT, and TPT) partially, but not significantly, suppressed AD production by the follicles at relatively high doses (Fig. 6A). The levels of AD exhibited considerable variation. When follicles were cultured in medium containing AD (100 ng/㎖), the amounts of T in the medium (control group) were in the range of 234-1,984 pg/follicle (n=6). As shown in Fig. 6B, all the phenyltins partially, but not significantly, suppressed the conversion of AD to T at high doses (> 10 μM). Taken together, these data showed that $C_{17\text{-}20}$ lyase and 17β-HSD in the follicles are not critically sensitive to the phenyltins.

DISCUSSION

In this study, we have demonstrated that some butyltins and phenyltins strongly suppressed FPH-induced P_5 and P_4 production by ovarian follicles of *R. dybowskii* and *R. catesbeiana in vitro*, probably through suppression of the P450scc enzyme. The other steroidogenic enzymes in the follicles such as 3β-HSD, $C_{17\text{-}20}$ lyase and 17β-HSD were much less sensitive to these organotins than P450scc and were not significantly suppressed by the organotins. 17α- hydroxylase was not affected by the organotins even at high doses. The toxic potential of butyltins followed the order TBT>DBT>TeBT>MBT. However, all the phenyltins (MPT, DPT, and TPT) exhibited similar toxicity to the follicular enzymes; they strongly suppressed P450scc and partially suppressed the other enzymes.

Organotin compounds are important commercial organometals, used widely as agricultural biocides, marine antifouling paints and industrial catalysts. Environmental contamination resulting from extensive use of organotin compounds has been of great concern because of their deleterious effects on non-target organisms including humans (Kimbrough, 1976; Maguire, 2000; Nielsen & Strand, 2002). With their increased usage, considerable attention has focused on potential toxicity of these compounds. When TPT accumulates in tissues of fish, it has been shown to inhibit cytochrome P-450scc in fish hepatoma cells (Bruschweiler et al., 1996; Ohhira & Matsui, 1996; Schwaiger et al., 1996). Previous studies demonstrated that phenyltin compounds (1 μM, *in vitro* exposure for 1 h) could cause considerable inhibition of the tumor-killing function of human NK cells. The toxic potential of the phenyltin compounds followed the order TPT> DPT> MPT (Whalen et al., 2000; Whalen et al., 2002). Human exposure to phenyltin compounds might come from occupational exposure

and/or consumption of contaminated food (Tolosa et al., 1992; Kannan et al., 1995; Ueno et al., 1999; Whalen et al., 2000). Natural killer (NK) cell are a primary immune defense against tumor and virally infected cell. Thus, phenyltin compounds may pose a threat of increased viral infection or tumorigenesis if they interfere with NK-cell function.

Several studies have reported that butyltin compounds suppressed steroidogenic enzyme activities. TBT directly inhibits the activities of steroidogenic P450scc and 3β-HSD at concentrations higher than 10 μM in bovine adrenal cultured cells (Yamazaki et al., 2005). It has also been reported that TBT at 12 μM and DBT at 74 μM inhibited human aromatase activity, and that MBT and TeBT were not suppressive (Cooke, 2002). TBT is also a competitive inhibitor of aromatase activity, with an IC_{50} value for 6.2 μM in human placental aromatase (Heidrich et al., 2001). McVey and Cooke (2003) found TBT to be the most potent inhibitor among butyltins of 3β-HSD activity in rat testis microsomes. These results indicate that butyltin compounds suppress steroidogenic enzyme activities in various mammalian cells, and that TBT is the most effective inhibitor. Previous study in our laboratory demonstrated that some kinds of butyltins (BTs) have inhibitory effects on the follicular steroidogenesis of *R. catesbeiana* (Ahn et al., 2007).

In this study, we have shown that the response of *R. dybowskii* ovarian follicles to organotin compounds is very similar to that of *R. catesbeiana*. Thus the data presented here and in our previous study (Ahn et al., 2007) demonstrate that some butyltins and phenyltins suppress steroidogenic enzymes, particularly P450scc, in *Rana* ovarian follicles *in vitro* as observed in fish and mammal. In general, the sensitivity of frog ovarian follicles cultured *in vitro* to butyltins appeared to be similar to or more sensitive than those obtained from various animal cell lines. For example, TBT suppressed conversion of cholesterol to pregnenolone (P450scc activity) at 1 μM in *R.* follicles *in vitro* (Fig. 2) while TBT suppressed the P450scc activity in bovine adrenal cultured cells at higher than 10 μM (Yamazaki et al., 2005). Because the frog follicle culture system is simple and cheap to maintain, it may provide a practical screening model to evaluate the potential effects of various endocrine disrupte(o)rs or environmental pollutants.

Population declines in some species of amphibians have been documented worldwide but causes of these declines remain unclear. Amphibians are vulnerable to water contamination because they have aquatic life histories and highly permeable skin (Pickford & Morris, 1999). Our study demonstrated that amphibian follicles are more sensitive to organotins (endocrine disruptors) than ovarian or testis cells of fish or mammals and this may be one of the reasons for the decline of amphibian species in nature. Further studies are needed to establish the amphibian follicle culture model as an experimental model for screening endocrine disruptors.

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