British Journal of Pharmacology (2003) 139, 1564–1570 
www.nature.com/bjp

# Direct effect of propylthiouracil on progesterone release in rat granulosa cells

[1]Jiann-Jong Chen, [2]Shyi-Wu Wang, [3]Eileen-Jea Chien & *,[3,4,5]Paulus S. Wang

[1]Department of Nursing, Cardinal Tien College of Nursing, Taipei 23148, Republic of China; [2]Department of Physiology and Pharmacology, College of Medicine, Chang Gung University, Kwei-Shan, Taoyuan 33333, Taiwan, Republic of China; [3]Department of Physiology, School of Medicine, National Yang-Ming University, Taipei 11221, Republic of China and [4]Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 11217, Republic of China

**1** The present study was to investigate the direct effect and action mechanism of propylthiouracil (PTU), an antithyroid drug, on the production of progesterone in rat granulosa cells.

**2** PTU (3–12 mM) decreased the basal and human chorionic gonadotropin (hCG)-stimulated release of progesterone from rat granulosa cells.

**3** PTU (3–12 mM) attenuated the stimulatory effects of forskolin and 8-bromo-cyclic 3′:5′-adenosine monophosphate on progesterone release from rat granulosa cells.

**4** PTU (12 mM) inhibited the activities of both the cytochrome *P*450 side-chain cleavage enzyme (*P*450scc, conversion of 25-hydroxyl cholesterol to pregnenolone) and the 3$\beta$-hydroxysteroid dehydrogenase (conversion of pregnenolone to progesterone) in rat granulosa cells. PTU decreased the $V_{max}$ but increased the $K_m$ of *P*450scc.

**5** PTU (12 mM) decreased the hCG-increased amount of steroidogenic acute regulatory (StAR) protein in rat granulosa cells.

**6** The present results suggest that PTU decreases the progesterone release by granulosa cells *via* a thyroid-independent mechanism involving the inhibition of post-cAMP pathway, and the activities of intracellular calcium, steroidogenic enzyme, and StAR protein functions.
*British Journal of Pharmacology* (2003) **139,** 1564–1570. doi:10.1038/sj.bjp.0705392

**Keywords:** Granulosa cells; progesterone; toxicology; propylthiouracil; calcium; *P*450scc activity; 3$\beta$-HSD activity; StAR expression

**Abbreviations:** 17$\beta$-HSD, 17$\beta$-hydroxysteroid dehydrogenase; 3$\beta$-HSD, 3$\beta$-hydroxysteroid dehydrogenase; 8-Br-cAMP, 8-bromo-adenosine 3′ : 5′-cyclic monophosphate; BSA, bovine serum albumin; hCG, human chorionic gonadotropin; 25-OH-cholesterol, 25-hydroxy-cholesterol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); LH, luteinizing hormone; *P*450arom, cytochrome *P*450 aromatase; *P*450scc, cytochrome *P*450 side-chain cleavage; PMSG, pregnant mares serum gonadotropin; PTU, propylthiouracil; StAR protein, steroidogenic acute regulatory protein

## Introduction

Propylthiouracil (PTU) and methimazole are antithyroid drugs that inhibit both the synthesis of thyroid hormones in thyroid gland (Cooper, 1984), and the deiodination of thyroxine ($T_4$) to its active form, triiodothyronine ($T_3$) (Cooper, 1984; Yang & Gordon, 1997). Clinical studies have reported that the most common side effect of PTU treatment in hyperthyroid patients is transient leukopenia, and in many cases, PTU has been found to induce severe toxic effects on the liver, including jaundice, severe hepatocellular dysfunction, and hepatomegaly (Jonas & Eidson, 1988; Levy, 1993; Deidiker & deMello, 1996).

It has been well documented that the reproductive endocrine systems in both male and female animals are affected by the PTU-induced hypothyroidism. In male rats, neonatal PTU-induced hypothyroidism reduces body weight, increases adult sperm production and testicular size by delaying maturation of Sertoli cells (De Franca *et al*., 1995; Hardy *et al*., 1996), and decreases serum testosterone (Mendis-Handagama *et al*., 1998). In male neonatal mice, PTU decreases the number of seminiferous tubules (Chan & Ng, 1995). In female prepubertal rats, administration of PTU resulted in a marked reduction of ovarian weight, and a decrease in the number of primordial, multilaminar, and Graafian follicles in mice (Chan & Ng, 1995). Our recent studies have demonstrated that PTU directly inhibits testosterone release by rat testicular interstitial cells (Chiao *et al*., 2000; 2002). Whether the alteration on female reproduction by PTU is partly due to a direct pharmacological or toxicological effect of PTU remains unknown.

In the present study, the effects of PTU on progesterone release by rat granulosa cells were examined. We found that the activities of both *P*450scc and 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD) were inhibited by PTU in granulosa cells. PTU decreased the expression of StAR protein which was

*Author for correspondence; E-mail: pswang@ym.edu.tw

[5]Current address: Department of Physiology, School of Medicine, National Yang-Ming University, Shih-Pai, Taipei, Taiwan 11221, Republic of China
Advance online publication: 7 July 2003

stimulated by hCG. These results suggest that the inhibitory effect of PTU on progesterone release is in part due to the reduction of the post-cAMP pathway, the activities of the steroidogenic enzymes, and the function of StAR protein.

## Methods

### *Reagents*

Chemicals and reagents including collagenase, hyaluronidase, pregnant mares' serum gonadotropin (PMSG), Dulbecco's modified Eagle's medium (DMEM)/F12, fatty acid-free bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), penicillin-G, streptomycin sulfate, insulin, medium-199 (M199), L-glutamine, 8-bromo-cAMP (8-Br-cAMP), 25-hydroxy-cholesterol (25-OH-cholesterol), pregnenolone, phenylmethylsulfonyl fluoride (PMSF), A23187, methimazole, and PTU were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile) was provided by Sanofi-Synthelabo, Inc. (Malvern, PA, U.S.A.). Cell culture plastic wares were obtained from Falcon Labware (Lincoln Park, NJ, U.S.A.). The antipregnenolone antiserum was purchased from Biogenesis (Poole, U.K.). The peroxidase-conjugated IgG fraction to mouse IgG and peroxidase-conjugated IgG fraction to rabbit IgG were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, U.S.A.). The anti-*P*450scc antibody and anti-StAR antibody were kindly provided by Dr B.C. Chung (Hu *et al*., 1991) and Dr D.M. Stocco (Lin *et al*., 1998), respectively.

### *Isolation and culture of granulosa cells*

Immature female Sprague–Dawley rats were housed in a temperature-controlled room ($22\pm1^{\circ}$C) with 14 h of artificial illumination daily (0600–2000 h) and were given food and water *ad libitum*. The preparation of granulosa cells was modified from the method described elsewhere (Tsai *et al*., 1999; Chen *et al*., 2001). The immature female rats, 22–25 days of age, were subcutaneously injected with PMSG (15 IU rat$^{-1}$). After 48 h, rats were killed by cervical dislocation. Ovaries were excised and transferred into the sterile DMEM F12$^{-1}$ (1 : 1) medium, containing 0.1% BSA, 20 mM HEPES, 100 IU penicillin-G ml$^{-1}$, and 50 $\mu$g streptomycin sulfate ml$^{-1}$. After trimming free fat and connective tissues, the large- and medium-sized follicles were punctured with a 26-gauge needle to release granulosa cells. The harvested cells were pelleted and resuspended in growth medium (DMEM F12$^{-1}$ containing 10% fetal calf serum, 2 $\mu$g insulin ml$^{-1}$, 100 IU penicillin ml$^{-1}$, and 100 $\mu$g streptomycin sulfate ml$^{-1}$). Cell viability was greater than 90% as determined using a hemocytometer and trypan blue method. Granulosa cells were aliquoted in the 24-well plates at approximately $1\times10^5$ cells well$^{-1}$ and incubated at 37°C with 5% $CO_2$–95% air for 2 days. Morphologically, the cultured granulosa cells maintained a characteristic round (or polygonal) shape, throughout our culture conditions.

### *Effects of PTU and MMZ on the release of progesterone by rat granulosa cells*

To ascertain the dose-dependent effects of PTU (3–12 mM) or MMZ (0.9–18 mM) in the presence or absence of hCG (0.5 IU ml$^{-1}$), or forskolin (an adenylyl cyclase activator, $10^{-6}$ M) or 8-Br-cAMP (a membrane-permeable analog of cAMP, $10^{-4}$ M), the granulosa cells were washed and incubated with 500 $\mu$l aliquots of serum-free BSA-M199 medium (M199 without phenol red, 0.3% BSA, 25 mM HEPES, 4 mM L-glutamine) containing different doses of PTU or MMZ with or without hCG, forskolin, and 8-Br-cAMP for 2 h at 37°C. The medium was collected and stored at −20°C until analysis for progesterone by radioimmunoassay (RIA).

### *Role of calcium in PTU effect on the release of progesterone by granulosa cells*

Granulosa cells were incubated with media containing PTU (1.5–12 mM) for 2 h in the presence or absence of A23187 ($10^{-5}$ M, a calcium inophore). After 2 h, the medium was collected and stored at −20°C until analysed for progesterone by RIA.

### *Effects of PTU on the activities of steroidogenic enzymes (cytochrome P450scc and 3β-HSD)*

Granulosa cells were incubated with media containing PTU (1.5–12 mM) for 2 h in the presence or absence of steroidogenic precursors including 25-OH-cholesterol ($10^{-5}$ M) and pregnenolone ($10^{-6}$ M). After 2 h, the medium was collected and stored at −20°C until analysed for progesterone or pregnenolone by RIA, respectively.

To further investigate the *P*450scc activity, granulosa cells were incubated with media containing PTU (12 mM) in the presence or absence of 25-OH-cholesterol ($10^{-5}$ M) and trilostane ($10^{-8}$–$10^{-5}$ M, an inhibitor of 3$\beta$-HSD). After 2 h, the medium was collected and stored at −20°C until analysed for pregnenolone by RIA.

For kinetic analysis of *P*450scc, granulosa cells ($2\times10^5$ cells well$^{-1}$) were primed with trilostane ($10^{-5}$ M) for 30 min and then incubated for 2 h with trilostane ($10^{-5}$ M) or trilostane plus 12 mM PTU in the presence of 25-OH-cholesterol ($10^{-8}$–$2.5\times10^{-6}$ M). At the end of incubation, the media were collected for pregnenolone RIA.

### *Effects of PTU on the expression of P450scc and StAR protein*

Granulosa cells were incubated with medium containing PTU (12 mM), in the presence or absence of hCG (0.5 IU ml$^{-1}$) for 2 h. After 2 h, cells were washed twice with saline and detached by trypsinization (1.25 mg ml$^{-1}$). The cells were collected and extracted in homogenization buffer (pH 8.0) containing 1.5% Na-lauroylsacrosine, $2.5\times10^{-3}$ M Tris-base, $1\times10^{-3}$ M EDTA, 0.68% PMSF, and 2% proteinase inhibitor cocktail, and then disrupted by ultrasonic sonicator (Heat Systems, Farmingdale, NY, U.S.A.) in an ice bath. Cell extracts were centrifuged at $13{,}500\times g$ for 10 min (Chen *et al*., 2001). The supernatant was collected and the protein concentration was determined by a colorimetric method for protein assay according to Bradford (1976).

### *Gel electrophoresis and Western blotting for P450scc and StAR protein expression*

Extracted proteins were denatured by boiling for 5 min in SDS buffer (0.125 M Tris-base, 4% SDS, 0.001% bromophenol

blue, 12% sucrose, and 0.15 M dithiothreitol) (Kau *et al*., 1999; Chen *et al*., 2001). The proteins (10 $\mu$g) in the samples were separated on 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) at 75 V for 15 min and then at 150 V for 40 min using a running buffer. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Inc., Boston, MA, U.S.A.) using a Trans-Blot SD semidry transfer cell (170-3940, Bio-Rad, Hercules, CA, U.S.A.) at 64 mA (for 8 mm × 10 mm membrane) for 45 min in a blotting solution. The membranes were washed in TBS-T buffer (0.8% NaCl, 0.02 M Tris-base, and 0.3% Tween-20, pH 7.6) for 5 min and then blocked by a 120 min incubation in blocking buffer (TBS-T buffer containing 5% nonfat dry milk). Then the membranes were incubated with a mixture of anti-*P*450scc antibodies (1 : 2000), anti-StAR protein antibodies (1 : 1000), and $\beta$-actin antibodies (1 : 2000) in 5% nonfat dry milk of TBS-T buffer overnight at 4°C. After one wash for 15 min and three washes for 5 min each time with TBS-T buffer, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (1 : 6000 dilution) and horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 8000 dilution) in 5% nonfat dry milk of TBS-T buffer. The membranes were washed four times with TBS-T buffer, and then the band for *P*450scc was visualized by chemiluminescence (ECL, Western blotting detection reagents, Amersham International, Buckinghamshire, U.K.).

### *Analysis of chemiluminescence Western blot data*

Quantification of chemiluminescence pseudo-autogradiograms on X-ray film was scanned using a scanner (Personal Densitometer, Molecular Dynamics, Sunyale, CA, U.S.A.). Quantification of scanned images was performed according to the user manual of the ImageQuaNT program (Molecular Dynamics, Sunyale, CA, U.S.A.). The P450scc and StAR protein signals are normalized to the $\beta$-actin signal, respectively.

### *RIAs of progesterone and pregnenolone*

The concentration of progesterone and pregnenolone in the medium was determined by RIA as described elsewhere (Chen *et al*., 2001). With antiprogesterone serum No. W5, the sensitivity of the progesterone RIA was 5 pg per assay tube. Intra- and interassay coefficients of variation (CVs) were 4.8% ($n=5$) and 9.5% ($n=4$), respectively. Antipregnenolone antiserum was diluted with 0.1% gelatin–PBS. The sensitivity of the pregnenolone RIA was 16 pg per assay tube. The inhibition curve produced by granulosa cell medium samples was parallel to that produced by pregnenolone. The intra- and interassay CVs were 2.3% ($n=6$) and 3.7% ($n=4$), respectively.

### *Statistical analysis*

All data were expressed as mean ± s.e.m. Treatment means were tested for homogeneity using the analysis of variance (ANOVA), and the differences between the specific means were tested for the significance by means of Duncan's multiple range test (Steel & Torrie, 1960). The chosen levels of significant and highly significant difference were $P<0.05$ and $P<0.01$, respectively.

## Results

### *Effects of PTU and MMZ on the release of progesterone by rat granulosa cells*

During a 2 h incubation, PTU (3–12 mM) elicited a dose-dependent inhibition of progesterone release by rat granulosa cells ($0.25\pm0.04$–$0.04\pm0.01$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, *versus* basal level $0.38\pm0.06$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.05$ or $P<0.01$, Figure 1). Incubation of granulosa cells with hCG (0.5 IU ml$^{-1}$), 8-Br-cAMP ($10^{-3}$ M), or forskolin ($10^{-6}$ M) for 2 h increased the level of progesterone secretion (hCG, $1.99\pm0.29$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$; 8-Br-cAMP, $0.98\pm0.08$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$ $P<0.01$; forskolin, $1.91\pm0.22$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$). Combination of hCG, 8-Br-cAMP, or forskolin with PTU (3–12 mM) resulted in a significant inhibition of the hCG-, 8-Br-cAMP- or forskolin-stimulated release of progesterone (hCG, $1.08\pm0.06$–$0.11\pm0.02$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$; 8-Br-cAMP, $0.70\pm0.09$–$0.07\pm0.01$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$; forskolin, $0.79\pm0.07$–$0.10\pm0.01$ ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 1). MMZ at same doses did not alter the basal, hCG-, 8-Br-cAMP- or forskolin-stimulated production of progesterone by rat granulosa cells (Figure 2).

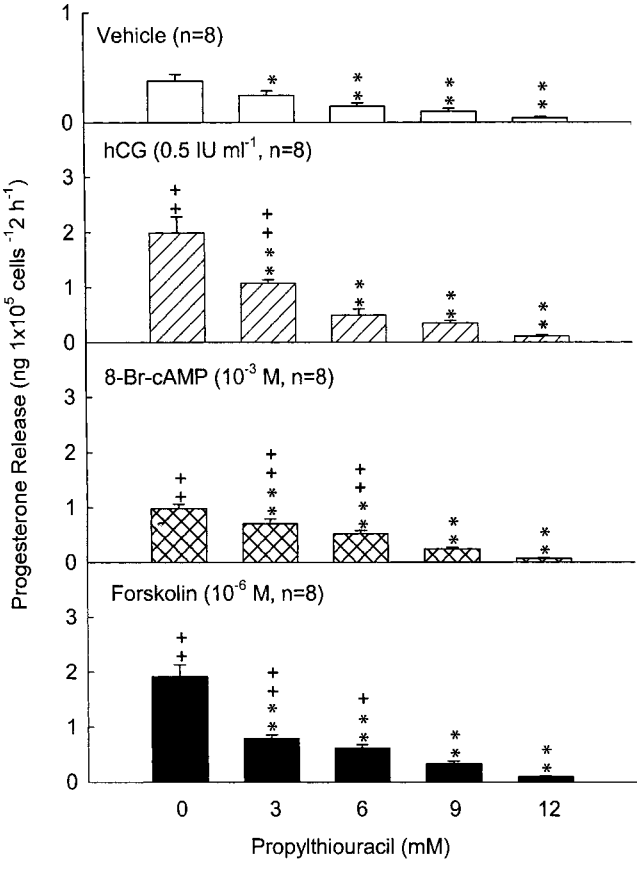


**Figure 1** Effects of different doses of PTU on the release of progesterone in the presence or absence of hCG (0.5 IU ml$^{-1}$), 8-Br-cAMP ($10^{-3}$ M), or forskolin ($10^{-6}$ M). *$P<0.05$, **$P<0.01$ compared with the value at PTU = 0 M, respectively. +$P<0.05$, ++$P<0.01$ compared with vehicle-treated group. Each column represents mean ± s.e.m.

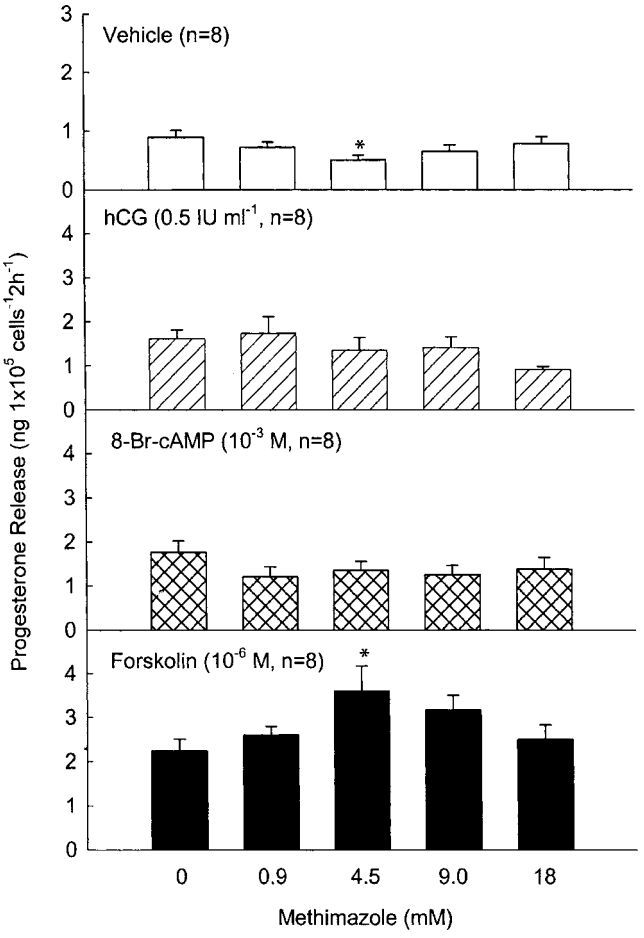


**Figure 2** Effects of different doses of methimazole on the release of progesterone in the presence or absence of hCG (0.5 IU ml$^{-1}$), 8-Br-cAMP ($10^{-3}$ M), or forskolin ($10^{-6}$ M). *$P<0.05$ compared with the value at methimazole = 0 M, respectively. Each column represents mean ± s.e.m.

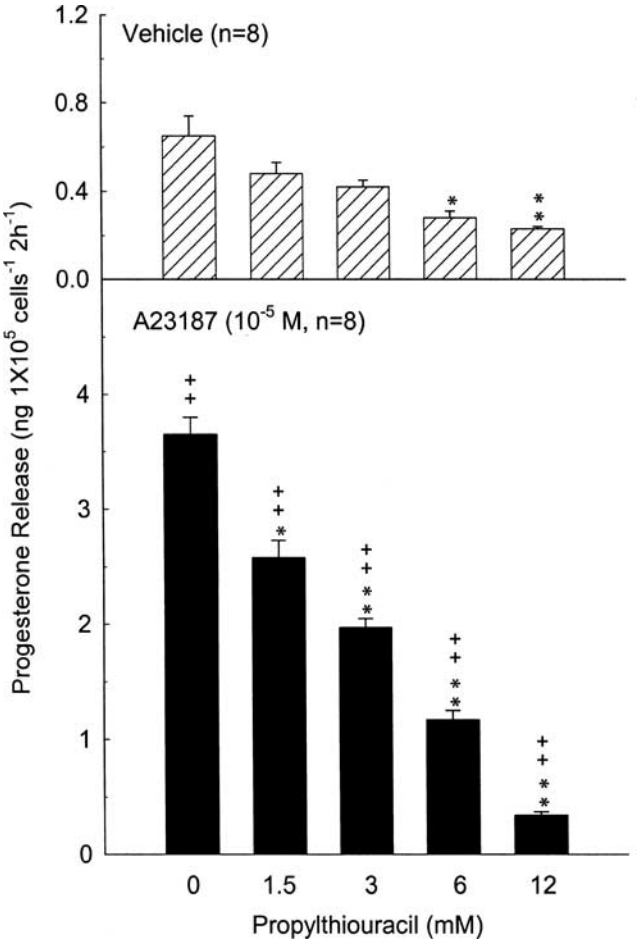


**Figure 3** Effects of different doses of PTU on the release of progesterone in response to A23187 ($10^{-5}$ M), *$P<0.05$, **$P<0.01$ compared with the value at PTU = 0 M, respectively, ++ $P<0.01$ compared with vehicle-treated group. Each column represents mean ± s.e.m.

### *Role of calcium in PTU effects on the release of progesterone by granulosa cells*

Administration of A23187 (a calcium ionophore, $10^{-5}$ M) stimulated the release of progesterone by rat granulosa cells (3.65 ± 0.15 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, *versus* vehicle group, 0.65 ± 0.09 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 4). PTU (1.5–12 mM) dose-dependently decreased both the basal and the A23187-stimulated release of progesterone by granulosa cells (basal level, 0.48 ± 0.09–0.23 ± 0.01 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.05$ or $P<0.01$; A23187-treated group, 2.58 ± 0.15–0.34 ± 0.03 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 3).

### *Effects of PTU on the activities of steroidogenic enzymes (cytochrome P450scc and 3β-HSD)*

Administration of 25-OH-cholesterol ($10^{-5}$ M) increased pregnenolone release (1.54 ± 0.21 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, *versus* vehicle group, 0.54 ± 0.14 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 4). PTU at 1.5–12 mM decreased not only the basal release of pregnenolone, but also the pregnenolone response to the 25-OH-cholesterol ($10^{-5}$ M) at PTU = 12 mM (basal level, 0.17 ± 0.05–0.06 ± 0.01 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$; 25-OH-cholesterol-treated group, 0.89 ± 0.10 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 4). Pregnenolone ($10^{-6}$ M) increased progesterone release (3.88 ± 0.44 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, *versus* vehicle group, 0.38 ± 0.06 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 4). PTU (1.5–12 mM) also decreased both the basal and the pregnenolone ($10^{-6}$ M)-stimulated production of progesterone (basal level, 0.25 ± 0.04–0.04 ± 0.01 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.05$ or $P<0.01$; pregnenolone-treated group, 1.13 ± 0.14–0.56 ± 0.06 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 4).

Using 3β-HSD inhibitor, trilostane ($10^{-8}$–$10^{-5}$ M), to inhibit the turnover of pregnenolone to progesterone significantly increased the production of pregnenolone in the presence or absence of 25-OH-cholesterol (25-OH-cholesterol = 0 M, 109.46 ± 7.82–429.26 ± 70.02 pg $10^5$ cells$^{-1}$ 2 h$^{-1}$, *versus* vehicle group 65.54 ± 12.73 pg $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$; 25-OH-cholesterol = $10^{-5}$ M, 296.44 ± 46.61–878.16 ± 168.76 pg $10^5$ cells$^{-1}$ 2 h$^{-1}$, *versus* vehicle group, 159.58 ± 49.09 pg $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 5). However, the inhibition of pregnenolone release by PTU (12 mM) was not reversed by the administration of trilostane

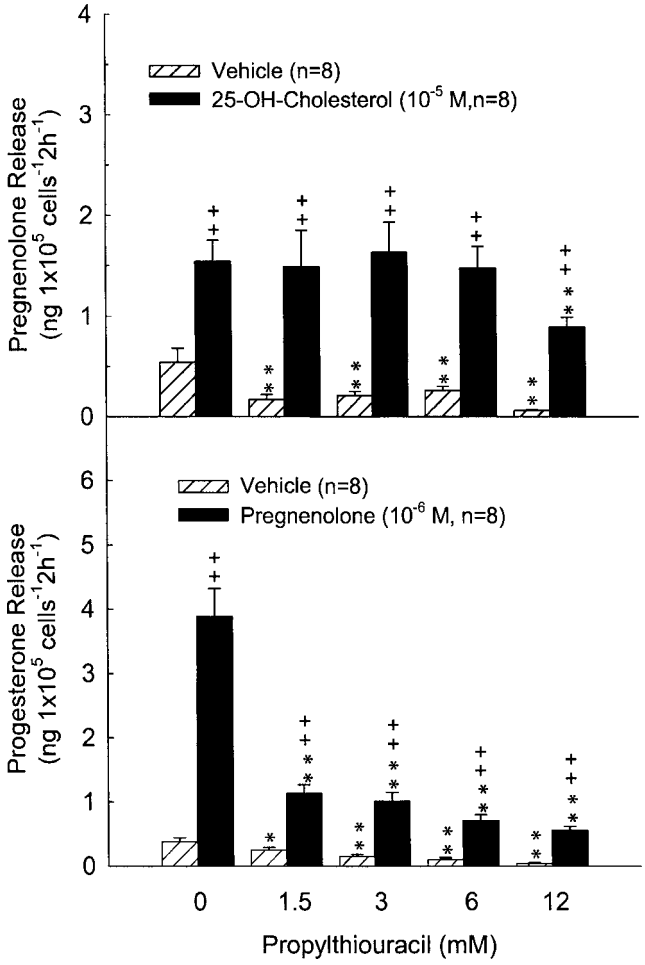


**Figure 4** Effects of PTU on the cytochrome *P*450scc enzyme and 3$\beta$-HSD activity in rat granulosa cells after incubation with 25-OH-cholesterol ($10^{-5}$ M) or pregnenolone ($10^{-6}$ M) for 2 h. $^*P<0.05$, $^{**}P<0.01$ compared with PTU = 0 M, respectively. $++P<0.01$ compared with 25-OH-cholesterol = 0 M or pregnenolone = 0 M, respectively. Each column represents mean ± s.e.m.

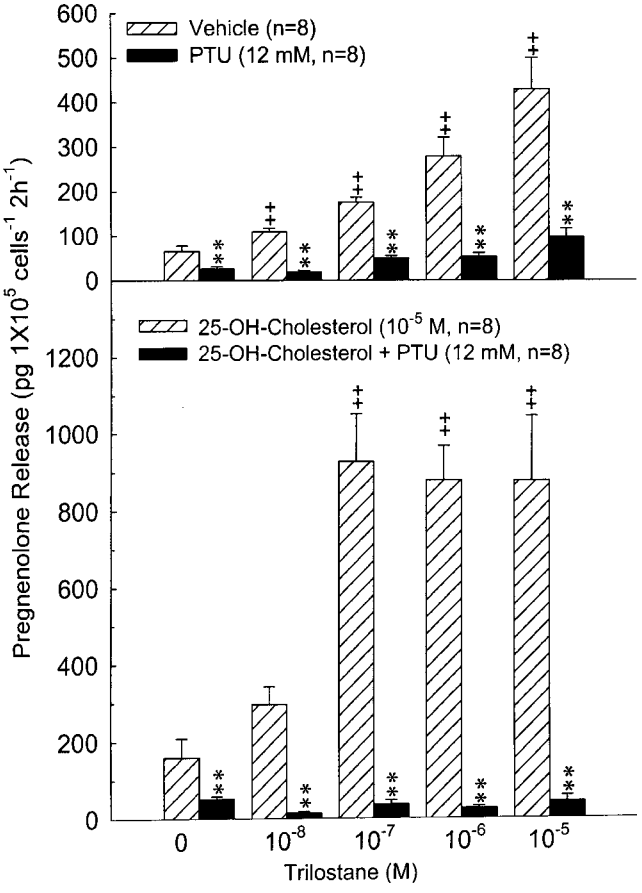


**Figure 5** Effects of PTU on the *P*450scc enzyme activity in rat granulosa cells after incubation with 25-OH-cholesterol ($10^{-5}$ M) and/or trilostane ($10^{-5}$–$10^{-6}$ M) for 2 h. $^*P<0.05$, $^{**}P<0.01$ compared with PTU = 0 M, respectively. $++P<0.01$ compared with trilostane = 0 M, respectively. Each column represents mean ± s.e.m.

($10^{-8}$–$10^{-5}$ M) as (25-OH-cholesterol = 0 M, 18.19 ± 4.06–95.57 ± 20.51 pg $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$; 25-OH-cholesterol = $10^{-5}$ M, 13.67 ± 4.04–42.32 ± 16.34 pg $10^5$ cells$^{-1}$ 2 h$^{-1}$, $n=8$, $P<0.01$) (Figure 5).

In kinetic study, the maximum velocities ($V_{max}$) were 4.81 ng h$^{-1}$ $2\times10^5$ cells$^{-1}$ in control group and 1.18 ng h$^{-1}$ $2\times10^5$ cells$^{-1}$ in PTU group. The Michaelis constants ($K_m$) were $0.11\times10^{-6}$ M in control group and $1.19\times10^{-6}$ M in PTU group (Figure 6).

### *Effects of PTU on the expression of* P450scc *and StAR protein*

Following 2 h incubation with rat granulosa cells, the resting amount of neither *P*450scc nor StAR protein was significantly altered by the administration of PTU (12 mM) (Figure 7). After the administration of hCG (0.5 IU ml$^{-1}$), the amount of StAR protein was increased by 50% ($P<0.05$, $n=8$), PTU (12 mM) inhibited the hCG-stimulated StAR protein expression ($P<0.01$) (Figure 7).

## Discussion

The present results demonstrated that PTU inhibited the spontaneous and hCG-stimulated secretion of progesterone and the activities of cytochrome *P*450scc and 3$\beta$-HSD by acting directly on rat granulosa cells.

Recently, we have demonstrated that PTU inhibits production of testosterone both *in vivo* and *in vitro* through the mechanisms involving a decrease of the basal and hCG-stimulated testosterone release, and an attenuation of the activities of cytochrome *P*450scc enzyme in rat testicular interstitial cells (Chiao *et al*., 2000; 2002). We also found that PTU, given acutely to euthyroid rats, decreases the rise in plasma corticosterone concentration induced by ACTH and corticosterone production *in vitro* in rat zona fasciculata-reticularis cells (Lo *et al*., 1998).

It has been well established that hCG stimulates progesterone secretion by granulosa cells and increases granulosa cAMP content (Sokka *et al*., 1996); cAMP activates protein kinase. The activated protein kinase modulates the activities of various steroidogenic enzymes, for example, cytocrome *P*450scc and 3$\beta$-HSD. On the other hand, gonadotropin depolarizes the membrane potential through cAMP elevation (Tsai *et al*., 1999). In the present study, we found that the basal release and hCG-stimulated production of progesterone by granulosa cells

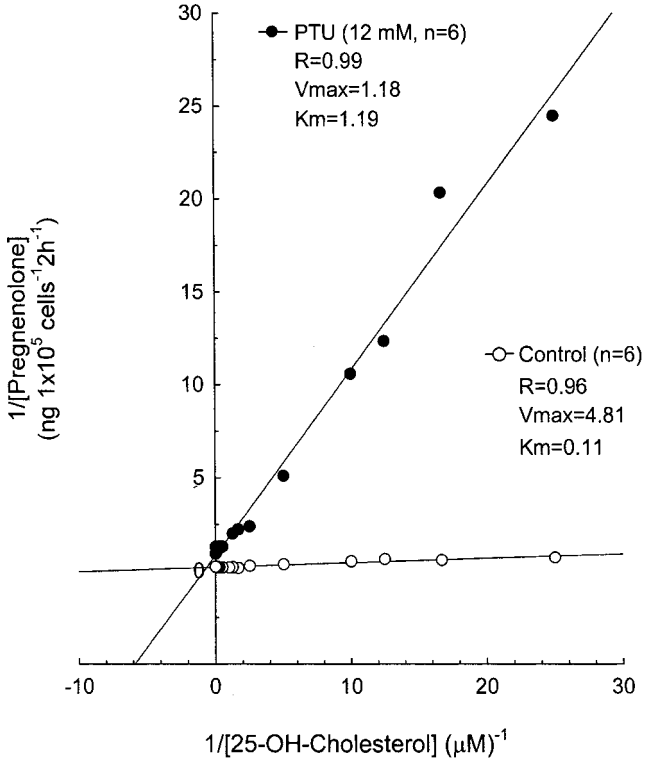


**Figure 6** Kinetic analysis of PTU inhibition of *P*450scc function. Double reciprocal plots of data were obtained from cultured granulosa cells challenged with 25-OH-cholesterol ($10^{-7}$–$10^{-4}$ M). The $V_{max}$ of the control group was 4.81 ng $2\times10^5$ cells$^{-1}$ 2 h$^{-1}$ and the PTU-treated group was 1. 18 ng $2\times10^5$ cells$^{-1}$ 2 h$^{-1}$, respectively. The $K_m$ of the PTU-treated group ($1.19\times10^{-6}$ M) was greater than that of the control group ($0.11\times10^{-6}$ M).

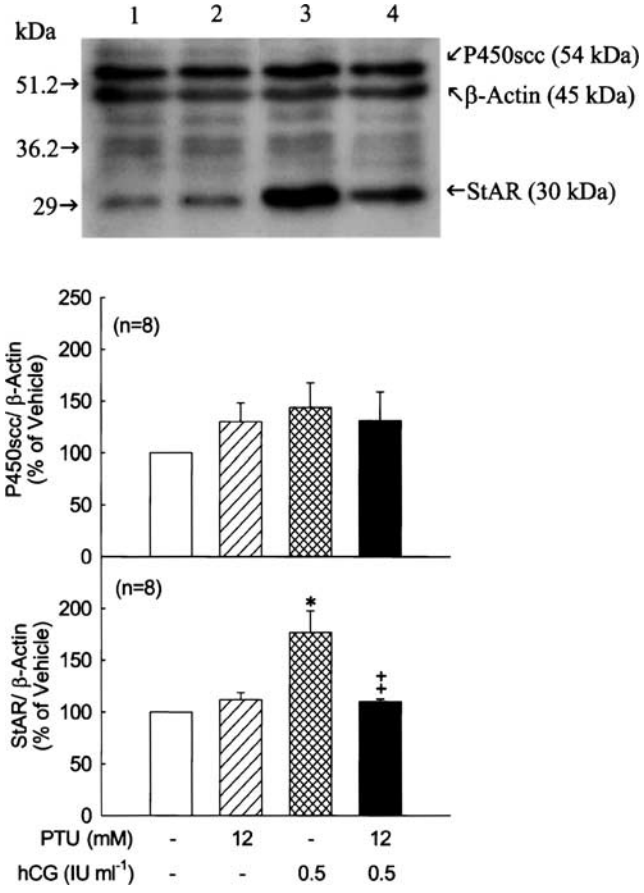


**Figure 7** Effects of PTU on the protein expression of cytochrome *P*450scc and StAR protein in rat granulosa cells. Rat granulosa cells were incubated with PTU (12 mM) or hCG (0.5 IU ml$^{-1}$) at 37°C for 2 h. Then, the cells were collected and analyzed by Western blotting. Each lane was loaded with 30 $\mu$g protein of sample. $^*P<0.05$ compared with the value at hCG = 0.05 IU ml$^{-1}$. $^+P<0.05$ compared with PTU = 0 M. Each column represents mean ± s.e.m.

were diminished by PTU (Figure 1), but not by methimazole (Figure 2). PTU and methimazole are well-known drugs for hyperthyroidism, which function by inhibiting both the synthesis of thyroid hormones in thyroid gland (Cooper, 1984), and the deiodination of thyroxine ($T_4$) to its active form, triiodothyronine ($T_3$) (Cooper, 1984; Yang & Gordon, 1997). These observations reflect the effects of PTU involving a thyroid-independent mechanism. Forskolin, an adenylyl cyclase activator, and 8-Br-cAMP, a membrane-permeable analog of cAMP, increased progesterone release, but neither of them reversed the inhibitory effects of PTU. These results suggest that PTU may directly act on the rat granulosa cells to regulate progesterone production and at a point distal to the formation of cAMP (Figure 1). The clinical dosage of PTU is 100–300 mg (Bromberg *et al*., 1992). In accordance with the molecular weight of PTU (MW = 170.2), the plasma concentration of PTU in patients was approximately 0.24–0.72 mM. The doses of PTU used in the present study (1.5–12 mM) seemed higher. However, PTU may provide an acute and/or accumulative effects in female gonads.

It has been reported that tetrandrine, a blocker for both L- and T-type $Ca^{2+}$ channels, decreases the $Ca^{2+}$ current in porcine granulosa cells (Kusaka *et al*., 1993). A23187 ($10^{-5}$ M) increased progesterone production by five folds (0.65 ± 0.09 to 3.65 ± 0.15 ng $10^5$ cells$^{-1}$ 2 h$^{-1}$). PTU (1.5–12 mM) decreased the progesterone release. A23187 reversed PTU-inhibited progesterone release (Figure 3). This result indicates that the effects of PTU are mediated partly *via* a calcium-dependent mechanism.

The final step in progesterone biosynthesis is the conversion of pregnenolone to progesterone under the catalyzation of the microsomal enzyme 3$\beta$-HSD (Hadley, 1995). We have demonstrated that pregnenolone at $10^{-8}$–$10^{-6}$ M stimulates progesterone release by rat granulosa cells (Chen *et al*., 2001), and reconfirmed this event in the present study. Since PTU (1.5–12 mM) attenuated the stimulatory effects caused by pregnenolone (Figure 4), it is apparent that PTU inhibited the activity of 3$\beta$-HSD, an enzyme for the conversion of pregnenolone to progesterone during the biosynthesis of progesterone. The stimulatory effect of 25-OH-cholesterol ($10^{-5}$ M) on the release of pregnenolone was also decreased by PTU. This suggests an inhibition of PTU on the activity of cytochrome *P*450scc enzyme (Figure 4), the rate-limiting enzyme for the conversion of cholesterol to pregnenolone during progesterone biosynthesis. To further identify the PTU effect on the *P*450scc activity, trilostane was used to block 3$\beta$-HSD activity. Trilostane ($10^{-8}$–$10^{-5}$ M) increased the pregnenolone accumulation, but the inhibitory effect of PTU was not reversed by trilostane (Figure 5).

The conversion of cholesterol to pregnenolone is the rate-limiting step in the final formation of progesterone and this

step is regulated by mitochondria enzyme *P*450scc (Waterman & Simpson, 1985). The rate-limiting step is the key point to decide the rate of progesterone formation. We measured the enzyme kinetic change of *P*450scc. In order to investigate the mechanism of PTU to inhibit *P*450scc function, we challenged granulosa cells with serial doses of 25-OH-cholesterol. The kinetic analysis of 25-OH-cholesterol-treated granulosa cells revealed that *P*450scc has an apparent $K_m$ of $0.11 \times 10^{-6}$ M and $V_{max}$ of 4.81 ng $2\,h^{-1}$ $2 \times 10^5$ cells$^{-1}$(Figure 6). The $V_{max}$ of PTU group (1.18 ng $2\,h^{-1}$ $2 \times 10^5$ cells$^{-1}$) was lower than that of control group, but the $K_m$, $1.19 \times 10^{-6}$ M, was almost 10-fold of the control value. This was consistent with either competitive or noncompetitive inhibition mechanism (Figure 6). PTU might interfere with the formation of binding complex of *P*450scc and cholesterol and decrease the rate of the conversion of cholesterol to pregnenolone *in situ* in granulosa cells. These results demonstrated that PTU inhibited the activity of *P*450scc in rat granulosa cells.

Based on our Western blot analysis, the amount of StAR protein was increased by the administration of hCG, but the stimulated effects were inhibited by PTU. The decreased StAR protein level suggests that the function of StAR protein in rat granulosa cells might be indirectly reduced by the action of PTU. On the other hand, the amount of cytochrome *P*450scc was not significantly altered by PTU. These results suggested that the decline of *P*450scc function was only due to the change of activity.

In summary, the present results demonstrate that PTU inhibits progesterone production by acting directly on rat granulosa cells. Even with the same doses as PTU, methimazole failed to affect progesterone production, which suggests that the inhibition was specific for PTU, not specific for granulosa cells. Taken together, the present data suggest that PTU decreases the progesterone release by granulosa cells *via* a thyroid-independent mechanism, which might involve the inhibition of post-cAMP pathway, calcium, cytochrome P450scc activity, 3$\beta$-HSD activity, and StAR protein expression.

The anti-*P*450scc antibody was kindly provided by Dr B.C. Chung, Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, ROC. The anti-StAR antibody was kindly provided by Dr D.M. Stocco, Department of Cell Biology and Biochemistry, Texas Tech University Health Science Center, Lubbock, TX, U.S.A. The technical assistance provided by Miss Jui-Ling Wang is appreciated.This study was supported by the grant No. NSC91-2320-B-010-047 from the National Science Council of the Republic of China.

## References

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.,* **72,** 248–254.

BROMBERG, N., ROMALDINI, J.H., WERNER, R.S., SGARBI, J.A. & WERNER, M.C. (1992). The evolution of Graves' ophthalmopathy during treatment with antithyroid drug alone and combined with triiodothyronine. *J. Endocrinol. Invest.,* **15,** 191–195.

CHAN, W.Y. & NG, T.B. (1995). Effect of hypothyroidism induced by propylthiouracil and thiourea on male and female reproductive systems of neonatal mice. *J. Exp. Zool.,* **273,** 160–169.

CHEN, J.J., WANG, P.S., CHIEN, E.J. & WANG, S.W. (2001). Direct inhibitory effect of digitalis on progesterone release from rat granulosa cells. *Br. J. Pharmacol.,* **132,** 1761–1768.

CHIAO, Y.C., LIN, H., WANG, S.W. & WANG, P.S. (2000). Direct effects of propylthiouracil on testosterone secretion in rat testicular interstitial cells. *Br. J. Pharmacol.,* **130,** 1477–1482.

CHIAO, Y.C., CHO, W.L. & WANG, P.S. (2002). Inhibition of testosterone production by propylthiouracil in rat testicular interstitial cells. *Biol. Reprod.,* **67,** 416–422.

COOPER, D.S. (1984). Antithyroid drugs. *N. Engl. J. Med.,* **311,** 1353–1362.

DE FRANCA, L.R., HESS, R.A., COOKE, P.S. & RUSSELL, L.D. (1995). Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. *Anat. Rec.,* **242,** 57–69.

DEIDIKER, R. & DEMELLO, D.E. (1996). Propylthiouracil-induced fulminant hepatitis: case report and review of the literature. *Pediatr. Pathol. Lab. Med.,* **16,** 845–852.

HADLEY, M.E. (1995). Hormones and female reproductive physiology. In *Endocrinology* ed. Hadley, M.E. pp. 476–504. Englewood Cliffs. NJ: Prentice-Hall.

HARDY, M.P., SHARMA, R.S., ARAMBEPOLA, N.K., SOTTAS, C.M., RUSSELL, L.D., BUNICK, D., HESS, R.A. & COOKE, P.S. (1996). Increased proliferation of Leydig cells induced by neonatal hypothyroidism in the rat. *J. Androl.,* **17,** 231–238.

HU, M.C., GUO, I.C., LIN, J.H. & CHUNG, B.C. (1991). Regulated expression of cytochrome *P*-450scc (cholesterol-side-chain cleavage enzyme) in cultured cell lines detected by antibody against bacterially expressed human protein. *Biochem. J.,* **274,** 813–817.

JONAS, M.M. & EIDSON, M.S. (1988). Propylthiouracil hepatotoxicity: two pediatric cases and review of the literature. *J. Pediatr. Gastroenterol. Nutr.,* **7,** 776–779.

KAU, M.M., CHEN, J.J., WANG, S.W., CHO, W.L. & WANG, P.S. (1999). Age-related impairment of aldosterone secretion in zona glomerulosa cells of ovariectomized rats. *J. Investig. Med.,* **47,** 425–432.

KUSAKA, M., TOHSE, N., NAKAYA, H., TANAKA, T., KANNO, M. & FUJIMOTO, S. (1993). Membrane currents of porcine granulosa cells in primary culture: characterization and effects of luteinizing hormone. *Biol. Reprod.,* **49,** 95–103.

LEVY, M. (1993). Propylthiouracil hepatotoxicity. A review and case presentation. *Clin. Pediatr.,* **32,** 25–29.

LIN, T., HU, J., WANG, D. & STOCCO, D.M. (1998). Interferon-gamma inhibited the steroidogenic acute regulatory protein messenger ribonucleic acid expression and protein levels in primary cultures of rat Leydig cells. *Endocrinology,* **139,** 2217–2222.

LO, M.J., WANG, S.W., KAU, M.M., CHEN, J.J., CHEN, Y.H., FANG, V.S., HO, L.T. & WANG, P.S. (1998). Pharmacological effects of propylthiouracil on corticosterone secretion in male rats. *J. Investig. Med.,* **46,** 444–452.

MENDIS-HANDAGAMA, S.M., ARIYARATNE, H.B., TEUNISSEN VAN MANEN, K.R. & HAUPT, R.L. (1998). Differentiation of adult Leydig cells in the neonatal rat testis is arrested by hypothyroidism. *Biol. Reprod.,* **59,** 351–357.

SOKKA, T.A., HAMALAINEN, T.M., KAIPIA, A., WARREN, D.W. & HUHTANIEMI, I. (1996). Development of luteinizing hormone action in the perinatal rat ovary. *Biol. Reprod.,* **55,** 663–670.

STEEL, R.D. & TORRIE, J.H. (1960). *Principles and Procedures of Statistics*, New York: McGraw-Hill.

TSAI, S.C., LU, C.C., CHEN, J.J., CHIAO, Y.C., WANG, S.W., HWANG, J.J. & WANG, P.S. (1999). Inhibition of salmon calcitonin on secretion of progesterone and GnRH-stimulated pituitary luteinizing hormone. *Am. J. Physiol.,* **277,** E49–E55.

WATERMAN, M.R. & SIMPSON, E.R. (1985). Regulation of the biosynthesis of cytochrome P450 involved in steroid hormone synthesis. *Mol. Cell. Endocrinol.,* **39,** 81–89.

YANG, Y. & GORDON, C.J. (1997). Regulated hypothermia in the hypothyroid rat induced by administration of propylthiouracil. *Am. J. Physiol.,* **272,** R1390–R1395.

*(Received January 25, 2003*
*Revised April 1, 2003*
*Accepted May 21, 2003)*