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Progesterone Attenuates the Inhibitory Effects of Cardiotonic Digitalis on Pregnenolone Production in Rat Luteal Cells

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Abstract Previous studies have shown that digoxin decreases testosterone secretion in testicular interstitial cells. However, the effect of digoxin on progesterone secretion in luteal cells is unclear. Progesterone is known as an endogenous digoxin-like hormone (EDLH). This study investigates how digitalis affected progesterone production and whether progesterone antagonized the effects of digitalis. Digoxin or digitoxin, but not ouabain, decreased the basal and human chorionic gonadotropin (hCG)-stimulated progesterone secretion as well as the activity of cytochrome P450 side chain cleavage enzyme (P450scc) in luteal cells. 8-Br-cAMP and forskolin did not affect the reduction. Neither the amount of P450scc, the amount of steroidogenic acute regulatory (StAR) protein, nor the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) was affected by digoxin or digitoxin. Moreover, in testicular interstitial and luteal cells, progesterone partially attenuated the reduction of pregnenolone by digoxin or digitoxin and the progesterone antagonist, RU486, blocked this attenuation. These new findings indicated that (1) digoxin or digitoxin inhibited pregnenolone production by decreasing the activity of P450scc enzyme, but not Na^+–K^+-ATPase, resulting in a decrease on progesterone secretion in rat luteal cells, and (2) the inhibitory effect on pregnenolone production by digoxin or digitoxin was reversed partially by progesterone. In conclusion, digoxin or digitoxin decreased progesterone production via the inhibition of pregnenolone by decreasing P450scc activity. Progesterone, an EDLH, could antagonize the effects of digoxin or digitoxin in luteal cells. J. Cell. Biochem. 86: 107–117, 2002. © 2002 Wiley-Liss, Inc.

Key words: corpus luteum; ovary; progesterone; steroid hormones; toxicology

Most cardiac glycosides are obtained from leaves of the foxglove, *Digitalis purpurea* or *Digitalis lanata* and have been used medicinally for several hundred years. Digoxin, digitoxin and related drugs elicit inotropic and chronotropic effects on the heart in patients with heart failure or atrial dysrhythmias [Antman and Smith, 1985]. The mechanism of action of digitalis involves a direct reversible inhibition of the Na^+–K^+-ATPase and permits sodium to remain in the cardiac cells, which is expelled using a sodium–calcium exchange process. The latter results in an increase in intracellular and myocardial calcium; this, in turn, increases the intracellular calcium, which leads to an increase of stroke for a given filling volume and pressure [Blanco and Mercer, 1998; Hauptman and Kelly, 1999].

The presence of a digoxin-like immunoreactive substance has been discovered at greater concentrations in umbilical cord blood samples than the corresponding maternal levels [Gonzalez et al., 1987]. The function of these substances may be reset to enhance cardiac performance during normal pregnancy [Gilson et al., 1997]. Ouabain or a closely-related isomer, and ouabain-like compounds, have been identified in plasma using several physico–chemical and biological methods [Hamlyn et al.,

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1989] and appears in blood while an animal is under the stress of hypoxia [De Angelis and Haupert, 1998]. Recently, ouabain in nontoxic concentrations has been shown to exert an effect similar to the endogeneous oubain-like putative hormone in cardiac myocytes and can regulate nonproliferative growth as well as the transcription of a number of growth-related genes [Peng et al., 1996; Xie et al., 1999; Haas et al., 2000]. Ouabain stimulates secondary messengers among which are intracellular Ca^{2+} and reactive oxygen species (ROS) [Liu et al., 2000].

It has been well known that progesterone is increased in essential hypertension [Seccombe et al., 1989], influences Na^+–K^+-ATPase in pregnant women [Longerich et al., 1988], and prevents ACTH-induced hypertension in sheep. Progesterone and its derivatives are reported to bind the digitalis receptor, Na^+–K^+-ATPase [LaBella, 1985]. Progesterone can efficiently displace ^{3}H-ouabain from Na^+–K^+-ATPase in the canine kidney [Seccombe et al., 1989]. The physiological level of progesterone found in pregnancy decreases Na^+–K^+-ATPase activity in the nephron of male rats [Mujais et al., 1985]. Apparently, progesterone, an endogenous digoxin-like hormone (EDLH), might be involved in regulating the biological activity of the Na^+–K^+-ATPase during stress or pregnancy.

It has been shown that the luteinizing hormone (LH)-increased steroidogenesis of progesterone is correlated with the increased generation of cAMP [Denning-Kendall and Wathes, 1994; Sekar et al., 2000]. Recently, the steroidogenic acute regulatory (StAR) protein has been reported to facilitate cholesterol transfer from the outer to the inner mitochondrial membrane [Reinhart et al., 1996; Stocco and Clark, 1996]. Cytochrome P450 side chain cleavage enzyme (P450scc), the rate-limiting enzyme for progesterone production, catalyzes cholesterol to pregnenolone in mitochondria of cells in ovary and other steroid-producing tissues [Waterman and Simpson, 1985; Dirami and Cooke, 1998]. The interconversion of pregnenolone, the common precursor of all steroid hormones, to progesterone is catalyzed by the microsomal enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) [Hadley, 1995].

Our previous studies have shown that digoxin decreases cAMP and testosterone secretion in rat testicular interstitial cells (TICs) [Lin et al., 1998a]. Digoxin and digitoxin decrease progesterone release in rat granulosa cells by reducing the expression of P450scc and the StAR protein [Chen et al., 2001]. However, the effects of digoxin or digitoxin on progesterone secretion in rat luteal cells are still not clear. In addition, there have been few reports investigating whether progesterone, an EDLH, exerts a protective role on cells to antagonize the effects of exogenous digitalis. Therefore, the aims of this study were to investigate how digitalis affected progesterone production and whether progesterone antagonized the effects of digitalis in rat luteal cells.

MATERIALS AND METHODS

Reagents

Chemicals and reagents including collagenase, hyaluronidase, pregnant mares serum gonadotropin (PMSG), Dulbecco's modified Eagle medium (DMEM)/F12, fatty acid-free bovine serum albumin (BSA), *N*-2-hydroxyethlypiperazine-*N*′-2-ethanesulphonic acid (HEPES), penicillin-G, streptomycin sulfate, insulin, medium-199 (M199), L-glutamine, 8-bromo-cAMP (8-Br-cAMP), 25-OH-cholesterol, digoxin, digitoxin, ouabain, pregnenolone, phenylmethylsulfonyl fluoride (PMSF), and RU486 were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture plasticware was obtained from Falcon Labware (Lincoln Park, NJ). The anti-pregnenolone antiserum was purchased from Biogenesis (Poole, UK). Trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile) was provided by Sanofi-Synthelabo, Inc. (Malvern, PA). The anti-pregnenolone antiserum was purchased from Biogenesis (Poole, UK). The peroxidase-conjugated IgG fraction to mouse IgG and peroxidase-conjugated IgG fraction to rabbit IgG were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH). The anti-P450scc 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., 1998b], respectively.

Isolation and Culture of Luteal Cells

Collagenase dispersion of luteal cells was modified from the method described previously [Roess et al., 1992]. The immature female rats with 25–27 days of age were intraperitoneal (i.p.) injected with PMSG (50 IU/rat). Forty-eight hours after PMSG treatment, the rats were injected (i.p.) with human chorionic gonadotropin (hCG) (25 IU/rat). One week later,

the rats were sacrificed, and the ovaries were collected and transferred into the sterilized physiological saline. After trimming free fat and connective tissues, the corpora lutea were weighed, chopped into 2–3-mm cubes, and washed with harvest medium (DMEM/F12 (1:1), containing 0.1% BSA, 20 mM HEPES, 100 IU/ml penicillin-G, 100 μg/ml streptomycin sulfate). Each gram of tissues was mixed with 5 ml of digest medium (DMEM/F12 (1:1), 0.1% BSA, 20 mM HEPES, 100 U/ml penicillin-G, 100 μg/ml streptomycin sulfate, 1,500 U collagenase/g tissue weight, 200 mg BSA/g tissue weight, 0.25% hyaluronidase), aerated with 95% O_2–5% CO_2, and then incubated and shaked (100 cycles/min) at 37°C for 1 h. During enzyme digestion, the tissues were repeatedly aspirated. The tissue clumps were allowed to settle. The suspension was collected, centrifuged at 300*g*, 20°C, for 10 min. The supernatant was discarded, and the precipitate was resuspended with culture medium (10% fetal bovine serum, 2 mg/ml insulin, 100 IU/ml penicillin G, 100 μg/ml streptomycin sulfate, 1:1 DMEM/F-12). Cell viability was greater than 90% as determined using a hemocytometer and trypan blue method. Luteal cells were aliquoted in 24-well plates at approximately 1×10^5 cells per well and incubated at 37°C with 5% CO_2–95% air for 1 day. Morphologically the cultured luteal cells maintained a characteristic round (or polygonal) shape throughout our culture conditions.

Isolation and Culture of Rat Leydig Cells

The method used for preparing the rat Leydig cells has been described elsewhere [Huang et al., 2001]. The male rats were decapitated, and the testes were collected. The medium (1% BSA in Hank's balanced salt solution with HEPES 25 mM, sodium bicarbonate 0.35 g/L, penicillin-G 100 IU/ml, streptomycin sulfate 50 μg/ml, heparin 2550 USP K U/L, pH 7.3) was aerated and saturated with 95% O_2 and 5% CO_2. Collagenase (Type IA, Sigma, St. Louis, MO) at a concentration of 700 μg/5 ml was used to disperse the TICs from the rat testis blocks [Tsai et al., 1997]. After filtration of rat testicular tissue, the filtrate containing TICs was centrifuged, washed, then resuspended in the medium to 5 ml, and then added gently to the upper layer of a continuous Percoll gradient. The continuous Percoll gradient (25 ml/dispersion) was composed of 9 parts of Percoll plus 11 parts of Medium 199 and 0.1% BSA before centrifugation at 4°C, 20,000*g* for 60 min. The mixture of TICs with continuous Percoll gradient was centrifuged at 4°C, 800*g* for 20 min. The Leydig cells layers were located in the layer 3–7 ml layers from the bottom of centrifuge tube. The Leydig cell layer was diluted to 5 ml and then centrifuged at 80*g*, at room temperature for 8 min. After repeating the wash step, the cell pellet was resuspended to 10 ml by the incubation medium. The concentration (1×10^5 cells/ml) and viability (over 95%) were determined using a hemocytometer and the trypan blue method.

Effects of Digoxin, Digitoxin, and Ouabain on the Release of Progesterone by Rat Luteal Cells

To ascertain the dose-dependent effects of ouabain (1×10^{-7}–1×10^{-5} M), digoxin (1×10^{-7}–1×10^{-5} M), and digitoxin (1×10^{-7}–1×10^{-5} M), in the presence or absence of hCG (0.5 IU/ml), on the production of progesterone, the luteal cells (1×10^5 cells/well) were washed and incubated with 500 μl 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 ouabain, digoxin, digitoxin with or without hCG for 2 h at 37°C. The medium was collected and stored at – 20°C until analysis for progesterone by RIA.

Effects of Digoxin, Digitoxin, and Ouabain on the Adenylyl Cyclase Activity and cAMP Action in Luteal Cells

Luteal cells (1×10^5 cells/well) were incubated with medium containing ouabain (1×10^{-5} M), digoxin (1×10^{-5} M), or digitoxin (1×10^{-5} M) at 37°C for 2 h in the presence or absence of forskolin (an adenylyl cyclase activator, 1×10^{-7}–1×10^{-5} M) or 8-Br-cAMP (a membrane permeable analog of cAMP, 1×10^{-5}–1×10^{-3} M). Two hours later, the medium was collected and stored at – 20°C until analyzed for progesterone by RIA.

Effects of Digoxin, Digitoxin, and Ouabain on the Activities of Steroidogenic Enzymes: Cytochrome P450scc Enzyme and 3β-HSD

Luteal cells (1×10^5 cells/well) were incubated with media containing ouabain (1×10^{-5} M), digoxin (1×10^{-5} M), or digitoxin (1×10^{-5} M) for 2 h in the presence or absence of steroidogenic precursors including 25-OH-cholesterol (1×10^{-8}–1×10^{-6} M) and pregnenolone (1×10^{-8}–1×10^{-6} M). Two hours later,

the medium was collected and stored at $-20^{\circ}C$ until analysis for progesterone by RIA.

To further investigate the P450scc activity, trilostane (a 3β-HSD inhibitor) was used. The luteal cells (1×10^5 cells/well) were incubated with media containing ouabain (1×10^{-5} M), digoxin (1×10^{-5} M), or digitoxin (1×10^{-5} M) for 2 h in the presence or absence of 25-OH-cholesterol (1×10^{-6}–1×10^{-5} M) and trilostane (1×10^{-7}–1×10^{-5} M). Two hours later, the medium was collected and stored at $-20^{\circ}C$ until analyzed for pregnenolone by RIA.

Effects of Digoxin, Digitoxin, and Ouabain on the Expression of P450scc and StAR Protein

Luteal cells (2×10^6/dish) were incubated with medium containing ouabain (1×10^{-5} M), digoxin (1×10^{-5} M), or digitoxin (1×10^{-5} M) for 2 h. Two hours later, the cells were washed twice with saline and detached by trypsinization (1.25 mg/ml). The cells were collected and extracted using homogenization buffer (pH 8.0) containing 1.5% Na-lauroylsacrosine, 2.5×10^{-3} M Tris-base, 1×10^{-3} M EDTA, 0.68% PMSF, and 2% proteinase inhibitor cocktail, and then disrupted using an ultrasonic sonicator (Heat Systems, Farmingdale, NY) in an ice-bath. The cell extracts were centrifuged at 13,500*g* for 10 min [Chen et al., 2001]. The supernatant was collected and the protein concentration was determined by a colorimetric method of protein assay [Bradford, 1976].

Effect of Progesterone on the Pegnenolone Release in Response to Digitalis in Rat Luteal Cells

Luteal cells (1×10^5 cells/well) were incubated with media containing ouabain (1×10^{-5} M), digoxin (1×10^{-5} M), or digitoxin (1×10^{-5} M) for 2 h in the presence or absence of progesterone (1×10^{-10}–1×10^{-6} M) at 37°C. Two hours later, the supernatant was collected and stored at $-20^{\circ}C$ until analysis for pregnenolone by RIA. The data on pregnenolone release by the vehicle group was taken as 100%, and the data for the drug-treated groups was presented as percentage of vehicle groups.

Effect of Progesterone on the Pregnenolone Release in Response to Digitalis in Rat Leydig Cells

A 90-min preincubation was followed by a challenged incubation. Rat Leydig cells (1×10^5 cells/tube) were incubated with medium containing ouabain (1×10^{-5} M), digoxin (1×10^{-5} M), or digitoxin (1×10^{-5} M) at 34°C for 2 h in the presence or absence of progesterone (1×10^{-10}–1×10^{-6} M). At the end of incubation, 2 ml of ice-cold gelatin phosphate buffer saline (0.1% gelatin in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.5) was added to stop the reaction. After centrifugation at 100*g* for 10 min, the supernatant was collected and stored at $-20^{\circ}C$ until analysis for pregnenolone by RIA. The data was analyzed as described above.

RU486 Reversed the Effect of Progesterone on Pregnenolone Release by Digoxin and Digitoxin in Rat Luteal Cells

Luteal cells (1×10^5 cells/well) were incubated with media containing digoxin (1×10^{-5} M), or digitoxin (1×10^{-5} M) for 2 h in the presence or absence of progesterone (1×10^{-6}–1×10^{-5} M) and RU486 (1×10^{-6}–1×10^{-5} M). Two hours later, the supernatant was collected and stored at $-20^{\circ}C$ until analysis for pregnenolone by RIA.

Gel Electrophoresis and Western Blotting for P450scc and StAR Protein Expression

Proteins in luteal cells were extracted and 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) [Chen et al., 2001]. The proteins (10 μg) 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 running buffer. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Inc., Boston, MA) using a Trans-Blot SD semi-dry transfer cell (170-3940, Bio-Rad, Hercules, CA) at 64 mA (for 8 mm × 10 mm membrane) for 45 min in 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 antibodies of anti-P450scc (1:2,000), anti-StAR protein (1:1,000), and anti-β-actin antibodies (1:2,000) 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 (1:6,000 dilution) and horseradish peroxidase-conjugated goat anti-mouse IgG

(1:8,000 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 P450scc, StAR and β-actin were visualized by chemiluminescence (ECL, Western blotting detection reagents, Amersham International, Buckinghamshire, UK).

Analysis of Chemiluminescence Western Blot Data

Quantification of the chemiluminescence pseudo-autoradiograms on X-ray film was carried out using a scanner (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA). Quantification of scanned images was performed according to the user manual of the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA). The P450scc and StAR protein signals were corrected by β-actin signal, respectively.

RIAs of Progesterone and Pregnenolone

RIA determined the amount of progesterone with anti-progesterone serum no. W5 [Chen et al., 2001]. The sensitivity of the progesterone RIA was 5 pg per assay tube. Intra- and inter-assay coefficients of variation (CV) were 4.8% (n = 5) and 9.5% (n = 4), respectively.

The amount of pregnenolone was determined by RIA [Chen et al., 2001]. Anti-pregnenolone antiserum (Biogenesis, Poole, UK) was diluted with 0.1% gelatin–PBS. The sensitivity of the pregnenolone RIA was 16 pg per assay tube. The inhibition curve produced by luteal cell medium samples was parallel to that produced by pregnenolone. The intra- and interassay CV were 2.3 (n = 6) and 3.7% (n = 4), respectively.

Statistical Analysis

All data were expressed as mean ± SEM. Treatment means were tested for homogeneity using 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 and Torrie, 1960]. The levels of significance were exhibited as significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively.

RESULTS

Effects of Digoxin, Digitoxin, and Ouabain on the Release of Progesterone in Rat Luteal Cells

The cross-reactivities of digoxin, digitoxin, and ouabain were less than 0.01% in the progesterone RIA system (data not shown). Digoxin at 5×10^{-6} or 1×10^{-5} M ($P < 0.05$ or < 0.01, respectively) and digitoxin at 5×10^{-7}–1×10^{-5} M ($P < 0.05$ or < 0.01, respectively) inhibited progesterone release by rat luteal cells after 2 h treatment (Fig. 1). Incubation of luteal cells with hCG (0.5 IU/ml) increased the level of progesterone secretion. Combination of hCG with digoxin at 5×10^{-6} or 1×10^{-5} M ($P < 0.05$ or < 0.01, respectively) or digitoxin at 1×10^{-7}–1×10^{-5} M ($P < 0.05$ or < 0.01, respectively) resulted in a significant inhibition of the hCG-stimulated release of progesterone (Fig. 1). Ouabain at the same doses did not affect the basal and hCG-stimulated production of progesterone by rat luteal cells.

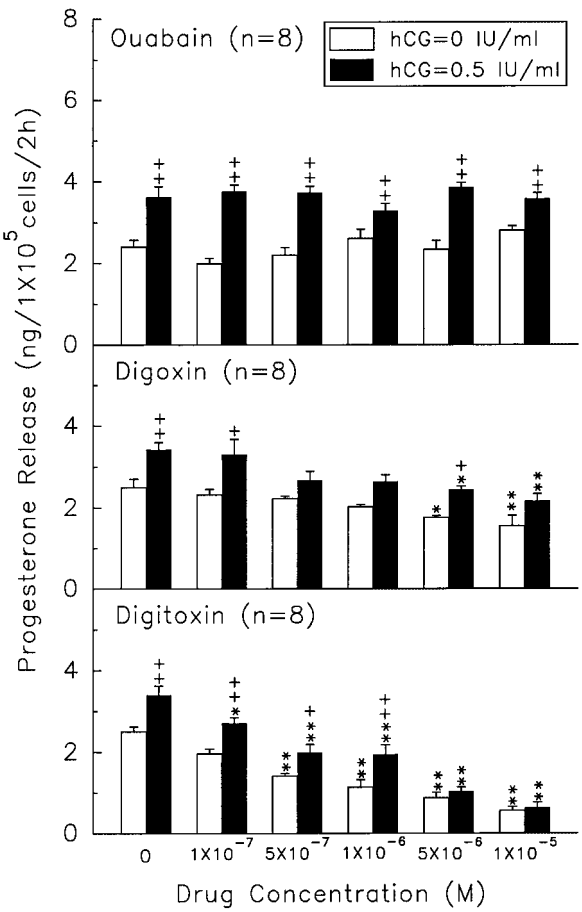

Fig. 1. Effects of different doses of ouabain (**top**), digoxin (**center**), and digitoxin (**bottom**) on the release of progesterone in the presence or absence of hCG (0.5 IU/ml). $*P < 0.05$, $**P < 0.01$ compared with the value at drug = 0 M, respectively. + $P < 0.05$, ++ $P < 0.01$ compared with the group treated without hCG, respectively. Each column represents mean ± SEM.

Effects of Digoxin, Digitoxin, and Ouabain on the Adenylyl Cyclase Activity and cAMP Action in Rat Luteal Cells

Forskolin (1×10^{-7}–1×10^{-5} M) stimulated the release of progesterone by rat luteal cells (Fig. 2A). However, digoxin or digitoxin at 1×10^{-5} M markedly decreased both the basal and forskolin-stimulated release of progesterone. In addition, ouabain at 1×10^{-5} M did not alter the stimulatory effects caused by 1×10^{-7} -1×10^{-5} M of forskolin (Fig. 2A).

Similar effects were obtained by using 8-Br-cAMP; at 1×10^{-4} and 1×10^{-3} M, 8-Br-cAMP stimulated the release of progesterone ($P < 0.01$) (Fig. 2B). 8-Br-cAMP did not reverse the inhibitory effects of digoxin (1×10^{-5} M) and digitoxin (1×10^{-5} M) (Fig. 2B). However, ouabain at 1×10^{-5} M did not alter the stimulation by 8-Br-cAMP (Fig. 2B).

Effects of Digoxin, Digitoxin, and Ouabain on the Activities of Steroidogenic Enzymes: Cytochrome P450scc and 3β-HSD

In order to understand which steroidogenic enzymes, cytochrome P450scc or 3-β-HSD, were affected by digoxin or digitoxin, 25-OH-cholesterol (1×10^{-6} M) and pregnenolone (1×10^{-8}–1×10^{-6} M) were administrated in luteal cells, and the results of significantly increased progesterone production were observed in both treatments ($P < 0.01$) (Fig. 3A,B). Digoxin (1×10^{-5} M) and digitoxin (1×10^{-5} M) not only decreased the basal release of progesterone, but also diminished the progesterone response to the 25-OH-cholesterol (1×10^{-6} M) ($P < 0.01$) (Fig. 3A). Pregnenolone, rather than 25-OH-

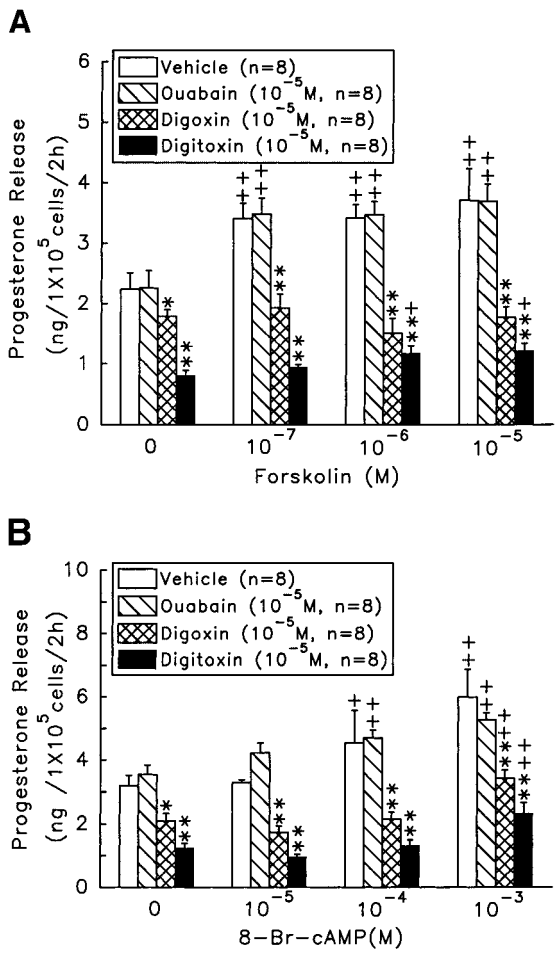

Fig. 2. Effects of ouabain, digoxin, and digitoxin on the release of progesterone in rat luteal cells in the presence of different doses of forskolin (**A**) and 8-Br-cAMP (**B**). *$P < 0.05$, **$P < 0.01$ compared with vehicle group, respectively. + $P < 0.05$, ++ $P < 0.01$ compared with 0 M of forskolin and 8-Br-cAMP, respectively. Each column represents mean ± SEM.

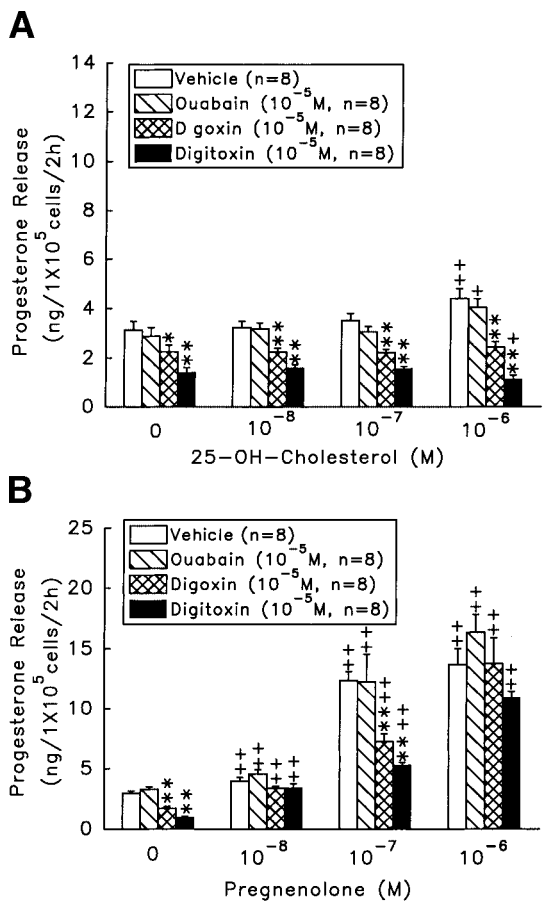

Fig. 3. Effects of ouabain, digoxin, and digitoxin on the release of progesterone in rat luteal cells in the presence of 25-OH-cholesterol (**A**) and pregnenolone (**B**). **$P < 0.01$ compared with vehicle group, respectively. + $P < 0.05$, ++ $P < 0.01$ compared with 0 M of 25-OH-cholesterol and pregnenolone, respectively. Each column represents mean ± SEM.

cholesterol, in the range of 1×10^{-8}–1×10^{-6} M could reverse the inhibitory effect by digoxin or digitoxin on progesterone release (Fig. 3B).

Digoxin (1×10^{-5} M) and digitoxin (1×10^{-5} M) caused a significant inhibition of pregnenolone release by the luteal cells ($P < 0.01$) as shown in Figure 4. In contrast, ouabain (1×10^{-5} M) did not change the release of pregnenolone. By using 3β-HSD inhibitor, trilostane (1×10^{-7}–1×10^{-5} M), to inhibit the turnover of pregnenolone to progesterone, ouabain (1×10^{-5} M) did significantly ($P < 0.01$) increase the releasing of pregnenolone. Whereas, the inhibition of pregnenolone release by digoxin and digitoxin was not reversed by administration with trilostane as vehicle controls (Fig. 4).

Effects of Digoxin, Digitoxin, and Ouabain on the Expression of P450scc and StAR Protein

When rat luteal cells were incubated with digoxin, digitoxin, or ouabain at 1×10^{-5} M for 2 h, the amounts of P450scc and StAR protein were not changed in cells (Fig. 5).

Effect of Progesterone on Pregnenolone Release in Response to Digitalis in Rat Luteal Cells

This experiment was to study whether progesterone antagonized the effects of digitalis in rat luteal cells. Digoxin and digitoxin at 1×10^{-5} M decreased the pregnenolone release in rat luteal cell shown as a percentage of the vehicle groups (Fig. 6). Progesterone in the range of 1×10^{-7}–1×10^{-6} M partially reversed the inhibitory effect of digoxin and digitoxin on pregnenolone release (Fig. 6).

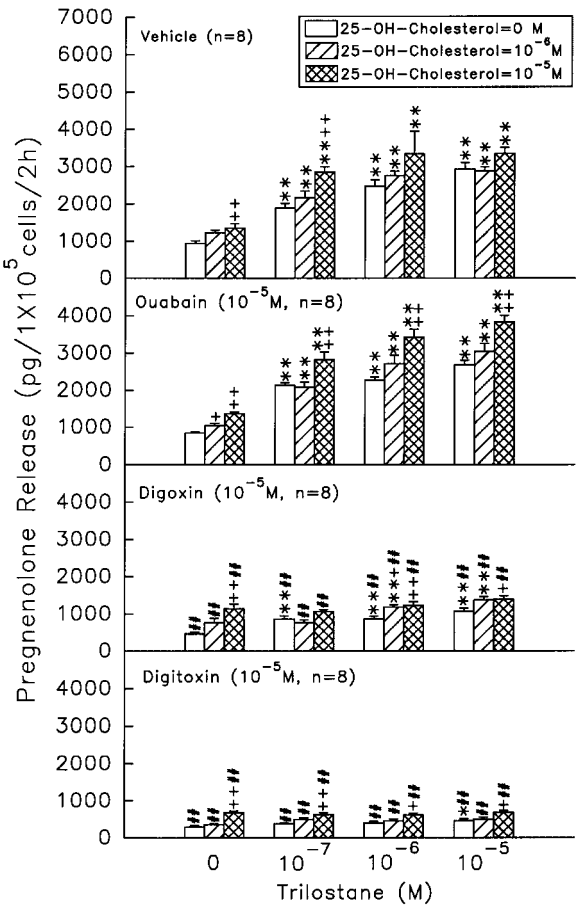

Fig. 4. Effects of ouabain, digoxin, and digitoxin on the cytochrome P450scc enzyme activity in rat luteal cells for 2 h. *$P < 0.05$, **$P < 0.01$ compared with 0 M of trilostane, respectively. + $P < 0.05$, ++ $P < 0.01$ compared with 0 M of 25-OH-cholesterol, respectively. ## $P < 0.01$ compared with 0 M of drug, respectively. Each column represents mean ± SEM.

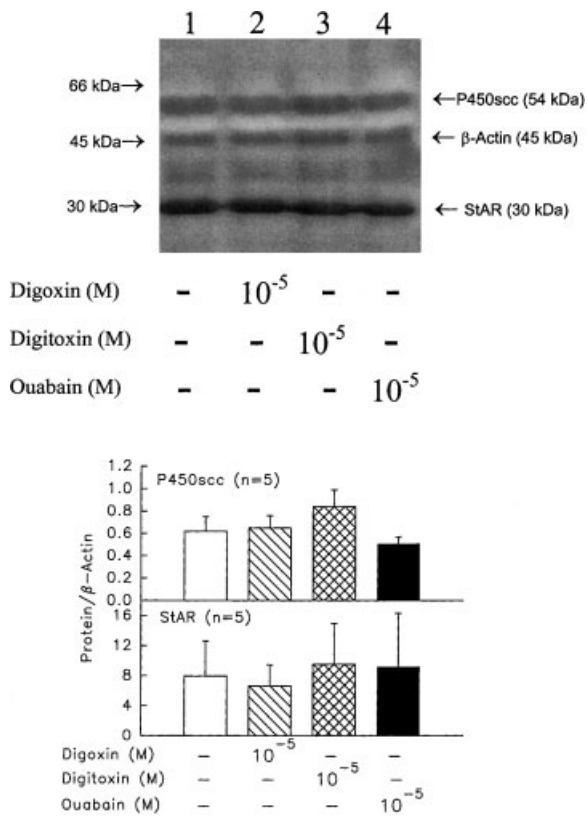

Fig. 5. Effects of ouabain, digoxin, and digitoxin on the protein amounts of cytochrome P450scc and StAR protein in rat luteal cells. Rat luteal cells were incubated with digoxin (1×10^{-5} M, **lane 2**), digitoxin (1×10^{-5} M, **lane 3**), or ouabain (1×10^{-5} M, **lane 4**), at 37°C for 2 h. Then, the cells were collected and analyzed by Western blotting. Each lane was loaded with 10 μg protein of sample. Similar results were obtained in four other experiments.

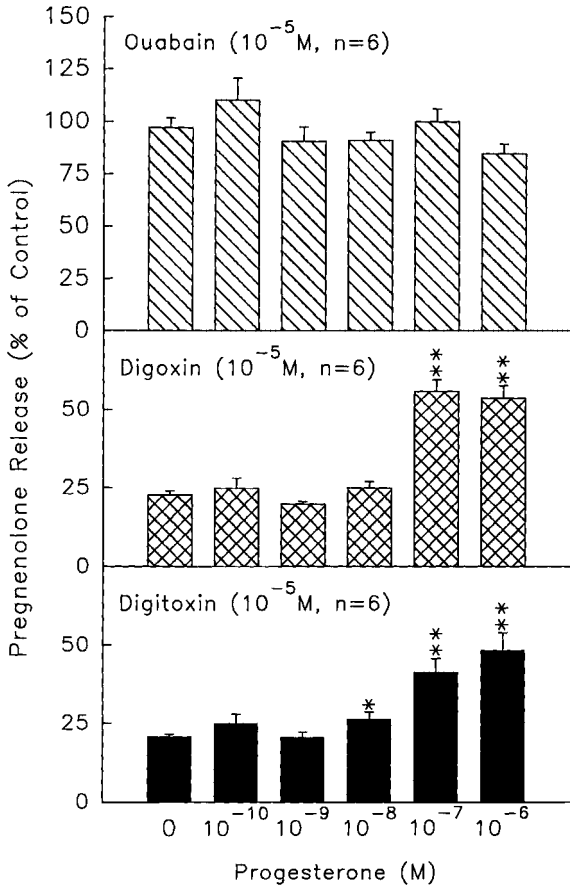

Fig. 6. Effects of ouabain (**top**), digoxin (**center**), and digitoxin (**bottom**) on the release of pregnenolone in rat luteal cells in the presence of different doses of progesterone (1×10^{-10}–1×10^{-6} M) at 37°C for 2 h. *$P < 0.05$, **$P < 0.01$ compared with 0 M of progesterone, respectively. Each column represents mean ± SEM.

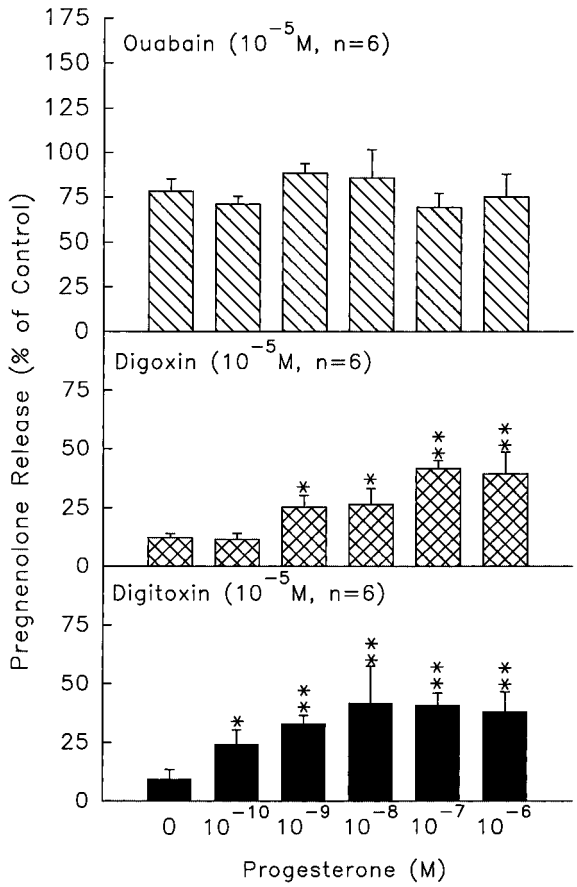

Fig. 7. Effects of ouabain (**top**), digoxin (**center**), and digitoxin (**bottom**) on the release of pregnenolone in rat Leydig cells in the presence of different doses of progesterone (1×10^{-10}–1×10^{-6} M) at 34°C for 2 h. *$P < 0.05$, **$P < 0.01$ compared with 0 M of progesterone, respectively. Each column represents mean ± SEM.

Effect of Progesterone on Pregnenolone Release in Response to Digitalis in Rat Leydig Cells

In order to confirm the effect of progesterone on pregnenolone release, the rat Leydig cells were used in present experiments. Digoxin and digitoxin at 1×10^{-5} M decreased the pregnenolone release from rat Leydig cells shown as a percentage of the vehicle groups (Fig. 7). Progesterone in the range of 1×10^{-9}–1×10^{-6} M did also partially reverse the inhibitory effect of digoxin and digitoxin on pregnenolone release (Fig. 7).

RU486 Reversed the Effect of Progesterone on Pregnenolone Release by Digoxin and Digitoxin in Rat Luteal Cells

Digoxin (1×10^{-5} M) and digitoxin (1×10^{-5} M) decreased the pregnenolone release from rat luteal cells compared to the vehicle group (Fig. 6). Progesterone in the range of 1×10^{-7} -1×10^{-6} M partially attenuated the inhibitory effect of digoxin and digitoxin on pregnenolone release in the medium (Fig. 8). The progesterone-reversed effects were significantly blocked by 1×10^{-5} M of RU486 ($P < 0.01$) (Fig. 8).

DISCUSSION

The digitalis steroids, digoxin, digitoxin, or ouabain did not cross-react with anti-progesterone serum (data not shown). The cardiotonic digitalis, digoxin, digitoxin, but not ouabain decreased the basal and hCG-stimulated progesterone secretion by rat luteal cells (Fig. 1). The decrease in progesterone was not attributed to the cytotoxicity of drugs. The administration 1×10^{-5} M of digoxin, digitoxin,

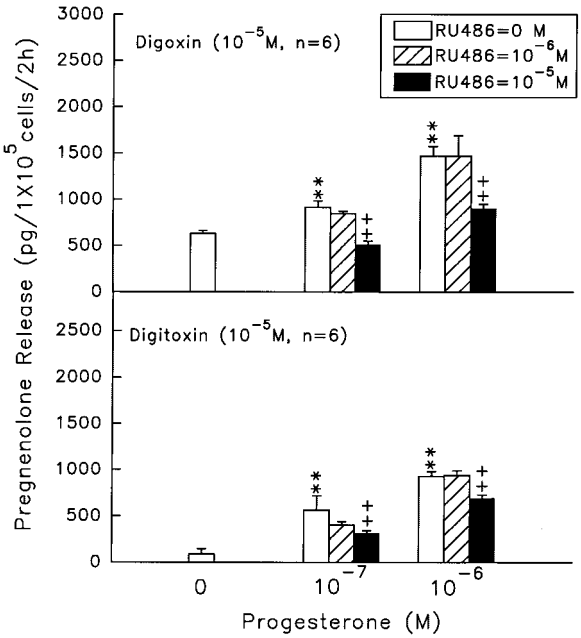

Fig. 8. Effect of RU486 on progesterone-prevented pregnenolone release by rat luteal cells. $^{**}P<0.01$ compared with the value at progesterone = 0 M, respectively. + $P<0.05$, ++ $P<0.01$ compared with progesterone = 1×10^{-7} or 1×10^{-6} M and RU486 = 0 M, respectively. Each column represents mean ± SEM.

or ouabain did not cause a releasing of lactate dehydrogenase (LDH) from luteal cells (data not shown). As shown in Figure 1, Na^+–K^+-ATPase inhibitor, ouabain, did not influence the progesterone secretion in luteal cells. For this reason, the decrease in progesterone secretion by digoxin, digitoxin was obviously not via the inhibition of the Na^+–K^+-ATPase. Thus, in the present study, ouabain was chosen as a drug of control to investigate how digoxin and digitoxin inhibited progesterone production in rat luteal cells.

The protein kinase C (PKC) inhibitors, sphingosine and psychosine, inhibit LH-stimulated cAMP accumulation and progesterone production in rat luteal cells, however, forskolin and cAMP analogues block these inhibitions. Thus, PKC is found to modulate steroidogenesis at a step before the cAMP synthesis in cells [Sender Baum and Ahren, 1988]. In the present study, digoxin and digitoxin were found to decrease progesterone secretion caused by forskolin or 8-Br-cAMP (Fig. 2). This fact indicated that the decrease of progesterone production by digoxin or digitoxin was due to neither a depression of the activity of the hormone-sensitive adenylate cyclase nor a lower level of cAMP formation, but to the inhibition of steps distal to cAMP formation. Pregnenolone, rather than 25-OH-cholesterol, reversed the inhibitory effects of digoxin and digitoxin on progesterone release (Fig. 3). We found that the steroidogenic steps in the conversion of 25-OH-cholesterol to pregnenolone by P450scc, but not pregnenolone to progesterone, by 3β-HSD were affected by digoxin or digitoxin (Fig. 3).

It was essential to investigate whether the regulation of the availability of free cholesterol by StAR protein or steroidogenic enzymes were affected by digoxin or digitoxin in cells. The phosphoprotein phosphatase inhibitors can decrease dibutyryl cAMP-stimulated progesterone secretion by inhibiting the availability of free cholesterol to mitochondria in rat luteal cells. Heat shock inhibits hormone-sensitive steroidogenesis in rat luteal cells and decreases synthesis of StAR protein as well as steroidogenesis in mouse Leydig cells [Liu and Stocco, 1997]. However, the Western blotting analysis of the enzyme P450scc and StAR proteins demonstrated that the level of expression of these two proteins in luteal cells was not reduced by digoxin or digitoxin (Fig. 5). Thus, we suggest that the decrease in progesterone secretion caused by digoxin and digitoxin is due to the decreased activity of the enzyme P450scc. The decrease of enzyme activity was further demonstrated by using trilostane, a 3β-HSD inhibitor (Fig. 4).

Progesterone was found to attenuate the inhibitory effects on P450scc activity caused by digoxin or digitoxin in luteal cells (Fig. 6). The treatment of progesterone would not increase the release of pregnenolone by oubain (Fig. 6). This illustrated that no cross-reaction existed between progesterone and anti-pregnenolone serum in the experiments. The similarity of inhibitory effects on P450scc activity was found in rat TICs by digoxin or digitoxin [Lin et al., 1998a]; thus, interstitial cells were used in this study to further confirm the existence of antagonized effect between progesterone and digoxin or digitoxin on P450scc activity (Fig. 7). Since digoxin or digitoxin has similar inhibitory effects on P450scc activity in rat TICs [Lin et al., 1998a], rat TICs were also employed to confirm the antagonized effect of progesterone on the inhibition of digoxin or digitoxin on P450scc activity (Fig. 7). Furthermore, we found that the progesterone antagonist RU486 prevented progesterone-attenuated

effects in luteal cells (Fig. 8). The above results indicated that progesterone might serve as an EDLH to reverse the effects of digoxin or digitoxin on gonadal function.

The corpus luteum, a unique tissue, secretes large amounts of progesterone to maintain pregnancy and does not express detectable levels of progesterone receptor (PR) mRNA [Park-Sarge et al., 1995]. Therefore, a classic PR is not required, but high specific binding sites are required for progesterone to exert its non-genomic actions in the rat corpus luteum during pregnancy. In the porcine corpus luteum, the rich specific progesterone binding sites are found to be associated with mitochondrial markers NADH-cytochrome C reductase in the presence of digitonin or saponin [Menzies and Bramley, 1994]. In this situation, digitonin is able to form a complex with sterols of the luteal membrane. According to the evidence, highly specific progesterone binding sites and its rate-limiting synthesis enzyme, P450scc, are both present in the mitochondria of luteal cells. In addition, in human granulosa cells, the antiprogesterone agent, RU486, inhibits the activity of enzyme P450scc. RU486 also inhibits LH or cAMP stimulated progesterone secretion [Parinaud et al., 1990]. Combination of the above facts and our findings imply that the enzyme P450scc might be another target protein in addition to Na^+–K^+-ATPase for progesterone or cardiotonic digitalis, digoxin, or digitoxin to act on. However, further studies will be necessary to examine how P450scc enzyme activity is inhibited by cardiotonic digitalis, digoxin, or digitoxin.

The activity of a digitalis-like factor rises throughout pregnancy and the return to normal levels after postpartum is believed to enhance cardiac performance during pregnancy [Gilson et al., 1997]. There are no reports, which study whether this endogenous digitalis-like factor can inhibit steroidogenesis in steroid-producing cells. In clinics, the iatrogenic cause of abnormal uterine bleeding by digitalis has been reported in women of reproductive ages and might result in complications in pregnancy. However, based on the present results, we suspected that high endogenous digoxin-like factor produced by the fetus to enhance cardiac performance of mother might severely hurt a normal pregnancy by a reduction in the progesterone level in blood. However, the generation of high levels of progesterone during pregnancy might serve as an overall physiological protection to avoid the endogeneous digitalis-like factor or exogenous dietary digitalis causing a decrease in the secretion of progesterone by luteal cells during pregnancy.

In summary, the findings of this study indicated that cardiotonic digitalis digoxin and digitoxin inhibited P450scc enzyme activity, but not Na^+–K^+-ATPase, resulting in a decrease in progesterone secretion by luteal cells. The inhibitory effect on P450scc activity induced by digitalis was partially reversed by the administration of progesterone. In conclusion, digoxin or digitoxin decreases progesterone secretion by inhibition of P450scc activity in luteal cells. The results suggested that progesterone might serve as an EDLH to attenuate the effects of digoxin or digitoxin on progesterone production.

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