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# Mechanisms of Digoxin and Digitoxin on the Production of Corticosterone in Zona Fasciculata-Reticularis Cells of Ovariectomized Rats

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**Abstract** Previous studies have indicated that digoxin (DG) inhibits testosterone production by rat testicular interstitial cells through both in vivo and in vitro experiments. DG and digitoxin (DT), but not ouabain, inhibit the progesterone, pregnenolone, and corticosterone secretion by rat granulosa cells, luteal cells, and zona fasciculata-reticularis (ZFR) cells, respectively. However, the effect of DG and DT on the enzyme kinetics of cytochrome P450 side chain cleavage enzyme (P450scc), the protein expression of P450scc and steroidogenic acute regulatory protein (StAR), and mRNA expression of StAR are unclear. ZFR cells were prepared from adrenocortical tissues of ovariectomized rats, and then challenged with adrenocorticotropin (ACTH), 8-Br-cAMP, forskolin, A23187, cyclopiazonic acid (CPA), nicotinic acid adenine dinucleotide phosphate (NAADP), trilostane, 25-OH-Cholesterol, progesterone, or deoxycorticosterone in the presence of DG, DT, or ouabain for 1 h. Enzyme kinetics of P450scc, protein expression of acute regulatory protein (StAR) and P450scc, and mRNA expression of StAR were investigated. DG and DT but not ouabain suppressed basal and other evoked-corticosterone release significantly. DG and DT also inhibited pregnenolone production. The $V_{max}$ of the DG and DT group was the same as the control group, but the $K_m$ was higher in DG- and DT-treated group than in control group. DT and ouabain significant suppressed mRNA expression of StAR. DG and DT had no effect on the P450scc and StAR protein expression at basal state, but diminished ACTH-induced StAR protein expression to basal level. These results indicated that DG and DT have an inhibitory effect on corticosterone production via a $Na^+$, $K^+$-ATPase-independent mechanism by diminishing actions on cAMP-, $Ca^{2+}$-pathway, competitive inhibition of P450scc enzyme and reduction of StAR mRNA expression. J. Cell. Biochem. 97: 303–313, 2006. © 2005 Wiley-Liss, Inc.

**Key words:** digoxin; digitoxin; corticosterone; zona fasciculata-reticularis cells; StAR; P450scc

Digoxin (DG) and digitoxin (DT) are two major components of digitalis. It has been known that these substances produce a profound beneficial effect on failing heart muscle. Indeed, DG and DT have widespread clinical use in the treatment of heart failure and atrial dysrhythmias [Antman and Smith, 1985; Demers et al., 1999]. The mechanism of digitalis action 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 later result is an increase in intracellular and myocardial calcium, which leads to increase stroke for a given filling volume and pressure [Blanco and Mercer, 1998; Hauptman and Kelly, 1999].

Patients receiving long term DG therapy show decreased plasma testosterone and luteinizing hormone levels [Stoffer et al., 1973; Neri et al., 1980] that may account for the inhibition of sexual desire and excitement observed in these patients. Therapy with digitalis glycosides

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has also been associated with gynaecourinary excretion of gonadotropin in postmenopausal women [Burckhardt et al., 1968], breast enlargement [Capeller et al., 1959], breast carcinoma [Stenkvist, 1999], and cornification of vaginal epithelium [Britsch and Azar, 1963; Navab et al., 1965] have been reported in several studies. Healthy men received therapeutic doses of DG for 43 days, the plasma cortisol concentration was no change [Kley et al., 1982]. Previously, we found that DG and DT inhibit the production of testosterone via a decrease of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in rat testicular interstitial cells [Lin et al., 1998; Wang et al., 1999]. Meanwhile, DG and DT decrease the progesterone release by granulosa cells via a $Na^+$, $K^+$-ATPase-independent mechanism involving the inhibition of post-cyclic AMP pathway, cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory (StAR) protein functions [Chen et al., 2001, 2002]. Recently, we have found that DG and DT but not ouabain decrease the release of corticosterone by acting directly on zona fasciculata-reticularis (ZFR) cells via a $Na^+$,$K^+$-ATPase-independent mechanism involving the inhibition of the activities of adenylyl cyclase, cytochrome P450scc and 11β-hydroxylase, as well as the functioning of cyclic AMP and intracellular calcium [Wang et al., 2004]. The conversion of cholesterol to pregnenolone is the rate-limiting step in the final formation of corticosterone and this step is regulated by mitochondrial enzyme P450scc [Waterman and Simpson, 1985; Dirami and Cooke, 1998]. However, the effects of DG and DT on the enzyme kinetic of P450scc, protein and mRNA expression of P450scc and StAR are still not clear.

In this study, we found that DG and DT inhibited adrenocorticotropin (ACTH)-stimulated release of corticosterone in rat adrenocortical cells, created competitive inhibition of P450scc enzyme and steroidogenic acute regulatory protein (StAR) mRNA expression. These results provide useful information on the regulation of anti-inflammation and glucose metabolism in response to digitalis.

## MATERIALS AND METHODS

### Animals

Female Sprague–Dawley rats (8–10 weeks) were purchased from National Yang-Ming University Animal Center. They were maintained under temperature ($22 \pm 1°C$) and light (0600–2000 h) controlled conditions with free access to food and water. Rats were bilaterally ovariectomized (ovx) under ether anesthesia and decapitated by a guillotine 14 days later. All animal experimentation has been conducted humanely and in conformance with policy statement of the Committee of National Yang-Ming University.

### Preparation of Adrenal Zona Fasciculata-Reticularis (ZFR) Cells

The method for the preparation of dispersed ZFR cells was modified from the method of Purdy et al. [1991] with minor modifications [Lo et al., 1998a,b]. Ovx rats were decapitated, and then the adrenal glands were rapidly excised and stored at an ice-cold 0.9% NaCl solution. After removal of excess fat, adrenal glands were separated into capsule (mainly zona glomerulosa) and inner zone (mainly ZFR) fractions. The inner zone from 8 to 10 adrenal glands were assigned as one dispersion, then the sliced fragments were incubated with collagenase (Sigma Chemical; 2 mg dissolved in 1 ml Krebs-Ringer bicarbonate buffer with 3.6 mmol $K^+$/L, 11.1 mmol glucose/L and 0.2% bovine serum albumin; pH 7.4; KRBGA) at 37°C in a shaking water bath for 1 h (100 cycles/min). At the end of incubation, the inner zone tissues were dispersed by repeated pipetting and filtered through a nylon mesh. After centrifugation (200*g*, 10 min), the supernatant was discarded, and the pellet was washed with deionized water for disrupting red blood cells; the osmolarity was then immediately restored with 10-fold Hank's balanced sodium solution (HBSS). The cell number was assessed in a hemocytometer after staining with trypan blue. The cells ($5 \times 10^4$ cells/0.5 ml) were preincubated with KRBGA medium for 1 h at 37°C in a shaker bath (100 cycles/min) aerated with 95% $O_2 + 5\% CO_2$.

### Drugs Treatment

The ZFR cells were incubated with 0.3 ml KRBGA medium, adrenocorticotropin (ACTH; $10^{-9}$ M), 8-bromo-cAMP (8-Br-cAMP, a permeable cAMP analog; $10^{-5}$ M), 3-isobutyl-1-methyl-xanthine (IBMX, a phosphodiesterase inhibitor; $5 \times 10^{-4}$ M), forskolin (an adenylyl cyclase activator; $10^{-5}$ M), angiotensin II (Ang II; $10^{-7}$ M), cyclopiazonic acid (CPA, a specific inhibitor of $Ca^{2+}$-ATPase in the sarcoplasmic

reticulum, and endoplasmic reticulum; $10^{-5}$ M), nicotinic acid adenine dinucleotide phosphate (NAADP, an activator of intracellular $Ca^{2+}$ release via an $IP_3$ and cyclic ADP ribose-independent mechanism; $10^{-5}$ M), A23187 (a $Ca^{2+}$ ionophore; $10^{-5}$ M), trilostane (3β-HSD inhibitor; $10^{-6}$ M), 25-OH-Cholesterol ($10^{-6}$ M), progesterone ($3 \times 10^{-7}$, $3 \times 10^{-5}$ M) or deoxycorticosterone (DOC, precursor of corticosterone; $3 \times 10^{-7}$, $3 \times 10^{-5}$ M) in the absence or presence of digitalis (DG, DT, or ouabain) ($10^{-6}$–$10^{-5}$ M) for 1 h. At the end of incubation, 0.2 ml ice-cold KRBGA medium was added to stop the incubation. The medium was centrifuged at 200*g* for 10 min and stored at −20°C, until it was analyzed for corticosterone and pregnenolone by radioimmunoassay (RIA).

### Western Blot Analysis

To determine the changes in the expression of P450scc and StAR protein, ZFR cells ($2 \times 10^6$ cells) were incubated with or without ACTH ($10^{-9}$ M), DG or DT ($10^{-5}$ M) for 2 h. At the end of incubation, cells were washed twice with ice-cold saline, and then homogenized, and the protein was extracted in 50 μl homogenization buffer (1.5% Na-lauroyl-sacrosine, 2.5 mM Tris base, 1 mM EDTA, 0.1% phenylmethyl sulfonylfluoride, pH 7.8). The cell extract was centrifuged at 12500*g* for 12 min at 4°C. The supernatant was collected and protein concentration was determined by a modification of the protein assay according to Bradford [Zor and Selinger, 1996].

Equal amount of proteins (40 μg per sample) was subjected to Western blot analysis. Blots were incubated with either anti-StAR (1:1,000), anti-P450scc (1:2,000) or anti-β-actin (1:8,000) antibodies overnight at 4°C. After washing three times (5 min each) in Tris Buffer Saline Tween-20 (20 mM Tris, 50 mM NaCl, 1% Tween-20, pH 7.6), the blots were incubated with a horseradish peroxidase-conjugated goat anti-rat secondary antibody (1:8,000) for 2 h at 4°C. After washing three times in TBST (5 min each), blots were visualized with a chemiluminescent detection system (ECL™ Western blotting detection reagents, Amersham International plc., Bucks, UK) as described by the manufacture.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

ZFR cells ($3 \times 10^6$ cells/ml) were incubated with or without DG, DT, or ouabain at $10^{-5}$ M for 30 min. At the end of incubation, the cells were washed twice and total RNA samples were isolated by TRIzol reagent-total RNA isolation reagent (Invitrogen, Carlsbad, CA). The procedures were conducted according to the manufacture's instructions. RNA samples were dissolved in water containing 0.1% diethylpyrocarbonate (DEPC), and quantified by measuring the absorbency at 260/280 nM. Aliquots containing 1 μg RNA were assayed by the relative-quantitative RT-PCR procedure, which was modified from the method described by Ronen-Fuhrmann et al. [1998]. RT was conducted for 2 h at 37°C using 250 ng pd(T) primer and 50 U of M-MuLV reverse transcriptase (BioLabs, Beverly, MA). PCR was performed in the presence of 1 μCi of [α-$^{32}$P]-deoxy-ATP (3000 Ci/mmol), dNTPs (mixture of dATP, dCTP, dGTP, and dTTP, 200 μM) and 500 ng appropriate oligonucleotide primers. Oligonucleotide primers for the ribosomal protein L19 served as an internal control [Ronen-Fuhrmann et al., 1998]. The number of cycles was examined to verify the amplification is in exponential phase. Following the PCR reaction (25 cycles), tracking dye was added to 10–20 μl of the PCR reaction mixture (90 μl) for analysis by 5% polyacrylamide gel electrophoresis [Orly et al., 1994]. The gels were dried and exposed to X-ray film and scanned by a scanner (Personal Densitometer, Molecular Dynamics, Sunyale, CA). Quantification of scanned images was performed according to ImageQuaNT™ program (Molecular Dynamics, Sunyale, CA). The radioactivity in each of the PCR bands was normalized to the radioactivity of the L19 band.

The PCR oligonucleotide primer pairs were designed based on known cDNA sequences of the various target genes. The expected PCR products would be 246 bp for the rat StAR cDNA [Ronen-Fuhrmann et al., 1998]; and 194 bp for the rat RPL19 [Orly et al., 1994]. Forward (A, sense) and reverse (B, antisense) primers were:

rat StAR A, 5′-GCAGCAGGCAACCTGGTG-3′
rat StAR B, 5′-TGATTGTCTTCGGCAGCC-3′
rat PRL19 A, 5′-CTGAAGGTCAAAGGGAAT-GTG-3′
rat PRL19 B, 5′-GGACAGAGTCTTGATGAT-CTC-3;

### RIA of Corticosterone and Pregnenolone

The concentration of corticosterone in the media was determined by RIA as described

elsewhere [Chen et al., 1997; Lo et al., 1998a]. With anticorticosterone No. PSW no. 4–9, the sensitivity of corticosterone was 5 pg per assay tube. The intra- and inter-assay coefficients of variation (CV) were 3.8% (n = 6) and 5.9% (n = 6), respectively.

The concentration of pregnenolone in the media was determined by RIA [Kau et al., 1999; Chang et al., 2002] with anti-pregnenolone antiserum purchased from Biogenesis, Inc (Sandown, NH). The sensitivity of the pregnenolone RIA was 16 pg per assay tube. The intra- and inter-assay CV were 2.9% (n = 6) and 5.5% (n = 6), respectively.

### Statistical Analysis

All data were expressed as mean ± SEM. Data were processed by one-way analysis of variance (ANOVA) using the SAS system. Multiple comparisons were performed where one-way ANOVA was significant, using the Duncan's multiple-range test [Steel and Torrie, 1980]. Differences between means were considered significant when $P < 0.05$, and highly significant when $P < 0.01$.

## RESULTS

### Effects of Digoxin (DG), Digitoxin (DT) and Ouabain on Corticosterone Secretion in ZFR Cells

Incubation of DG and DT ($10^{-6}$ or $10^{-5}$ M) for 1 h resulted in a significant ($P < 0.05$ or $P < 0.01$) decrease in corticosterone secretion in a dose-dependent manner (Fig. 1, upper panel). Ouabain at the same doses did not affect the basal release of corticosterone by rat ZFR cells. Administration of ACTH ($10^{-9}$ M) or 8-Br-cAMP ($10^{-5}$ M) alone markedly ($P < 0.01$) increased corticosterone secretion, as compared with the basal group, and this stimulatory effect was attenuated by DG and DT ($10^{-6}$–$10^{-5}$ M) but not ouabain in a dose-dependent manner ($P < 0.05$ or $P < 0.01$) (Fig. 1, middle and lower panel).

### Effects of DG, DT, and Ouabain on the cAMP Function in ZFR Cells

Incubation of ZFR cells with forskolin ($10^{-5}$ M) for 1 h produced a significant increase in corticosterone secretion ($P < 0.01$) (Fig. 2). Corticosterone responses to forskolin were reduced significantly when DG and DT ($10^{-5}$ M) ($P < 0.05$ or $P < 0.01$) but not ouabain were treated in the medium.

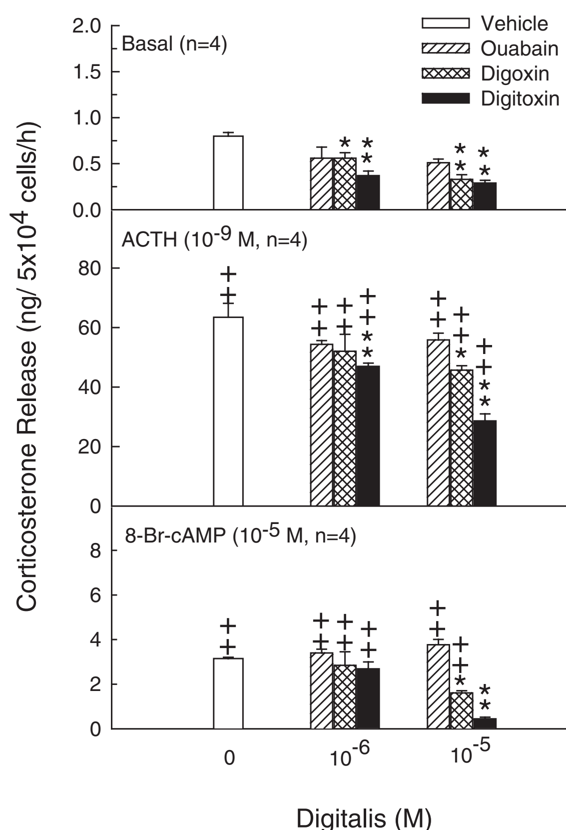


Fig. 1. Effects of digoxin (DG), digitoxin (DT) and ouabain ($10^{-6}$–$10^{-5}$ M) on corticosterone release by ovx rat ZFR cells treated with or without ACTH ($10^{-9}$ M) and 8-Br-cAMP ($10^{-5}$ M). Each column represents mean ± SEM. *, **: $P < 0.05$, $P < 0.01$ compared with corresponding vehicle group. ++: $P < 0.01$ compared with basal group.

In the presence of IBMX (phospshodiesterase inhibitor; $5 \times 10^{-4}$ M), forskolin produced an approximately 12-fold increase in corticosterone secretion in ZFR cells ($P < 0.01$). These responses were reduced significantly when DG and DT ($10^{-5}$ M) ($P < 0.05$ or $P < 0.01$) were added in the medium.

### Effects of DG and DT on the Activities of Intracellular Calcium

Administration of angiotensin II (Ang II; $10^{-7}$ M), cyclopiazonic acid (CPA, $10^{-5}$ M; a specific inhibitor of $Ca^{2+}$-ATPase in the sarcoplasmic reticulum and endoplasmic reticulum), NAADP ($10^{-5}$ M) (Fig. 3), or A23187 ($10^{-5}$ M; a calcium ionophore)(Fig. 3) produced a significant ($P < 0.05$ or $P < 0.01$) increase in corticosterone release from ZFR cells, and this stimulatory effect was attenuated by DG and DT ($10^{-6}$–$10^{-5}$ M) ($P < 0.05$ or $P < 0.01$).

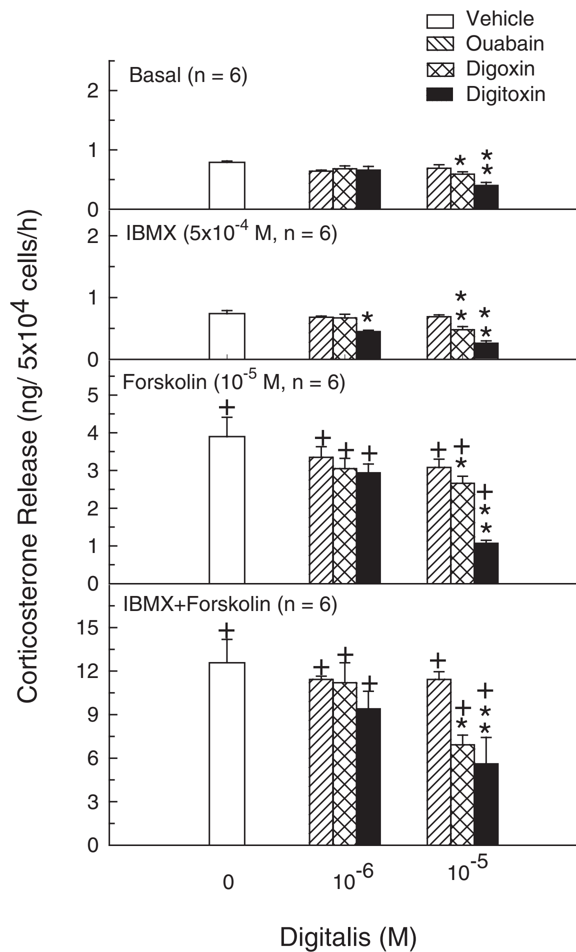


**Fig. 2.** Effects of DG, DT, and ouabain ($10^{-6}$–$10^{-5}$ M) on corticosterone release by ovx rat ZFR cells treated with or without IBMX ($5 \times 10^{-4}$ M) and forskolin ($10^{-5}$ M). Each column represents mean ± SEM. *, **: $P < 0.05$, $P < 0.01$ compared with corresponding vehicle group. +: $P < 0.01$ compared with basal group.

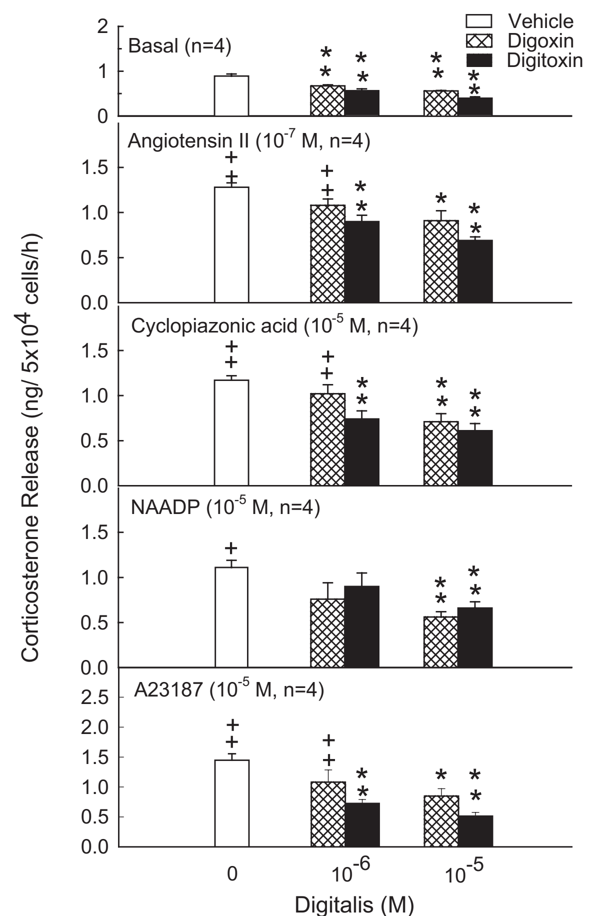


**Fig. 3.** Effects of DG and DT ($10^{-6}$–$10^{-5}$ M) on corticosterone release by ovx rat ZFR cells treated with or without angiotensin II ($10^{-7}$ M), cyclopiazonic acid (CPA) ($10^{-5}$ M), NAADP ($10^{-5}$ M), and A23187 ($10^{-5}$ M). Each column represents mean ± SEM. *, **: $P < 0.05$, $P < 0.01$ compared with corresponding vehicle group. +, ++: $P < 0.05$, $P < 0.01$ compared with basal group.

## Effects of DG and DT on the Activities of Steroidogenic Enzymes

Administration of 25-hydroxy-cholesterol ($10^{-7}$, $10^{-5}$ M; membrane permeable cholesterol, 25-OH-Cholesterol), pregnenolone ($3 \times 10^{-7}$, $3 \times 10^{-5}$ M; the precursor of progesterone) (Fig. 4), or progesterone ($3 \times 10^{-7}$, $3 \times 10^{-5}$ M; the precursor of deoxycorticosterone) (Fig. 5) significantly increased corticosterone production in a dose-dependent manner. Coincubation of the steroidogenic precursors with DG or DT ($10^{-5}$ M) resulted in a decrease of corticosterone production except progesterone. The effects of DG and DT ($10^{-5}$ M) on 11β-hydroxylase to inhibit corticosterone secretion have been examined. Administration of deoxycorticosterone ($3 \times 10^{-7}$, $3 \times 10^{-5}$ M) markedly ($P < 0.01$) increased corticosterone secretion, as compared with the unstimulated group, and attenuated by DT ($10^{-5}$ M) (Fig. 5). For further studying the change of P450scc activity, an inhibitor of 3β-HSD, trilostane, was employed. The release of pregnenolone from ZFR cells was increased in the presence of trilostane ($10^{-6}$ M), 25-OH-Cholesterol ($10^{-6}$ M) or trilostane plus 25-OH-Cholesterol ($P < 0.05$) (Fig. 6). DG and DT ($10^{-5}$ M) attenuated the stimulatory effect caused by trilostane, 25-OH-Cholesterol, or trilostane plus 25-OH-Cholesterol. The maximum velocities ($V_{max}$) were almost the same among the control, DG and DT (Fig. 7) groups. The Michaelis constant ($K_m$) was 0.32, 1.37 and 2.03 μM, respectively (Fig. 7).

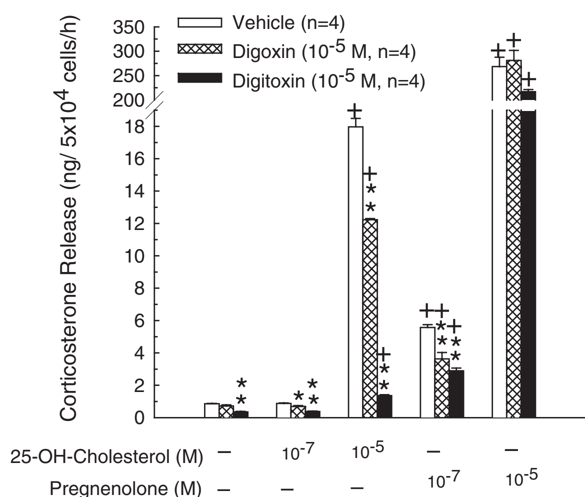


**Fig. 4.** Effects of DG and DT ($10^{-6}$–$10^{-5}$ M) on corticosterone release by ovx rat ZFR cells treated with or without 25-OH-Cholesterol and pregnenolone ($10^{-7}$ or $10^{-5}$ M). Each column represents mean ± SEM. **: $P < 0.01$ compared with corresponding vehicle group. +: $P < 0.01$ compared with basal group.

## Effects of DG and DT on the Protein and Messenger RNA Expression of StAR and P450scc

To determine whether the inhibitory effects of DG and DT were caused by altered expressions of StAR, P450scc protein and messenger RNA, after administration of DG, DT, ouabain ($10^{-5}$ M), and ACTH ($10^{-9}$ M) for 2 h or 30 min, respectively, the levels of these proteins in ZFR cells were assessed by Western blotting (Fig. 8), and the levels of StAR mRNA expression were assessed by RT-PCR (Fig. 9), respectively. In our results, signal of β-actin (45 kDa) and PRL 19 (194 bp) were used as an internal control. Bands at 54 kDa (P450scc) and 30 kDa (StAR) molecular weight were detected in rat adrenal ZFR cells. Based on the ratio of internal control, neither P450scc nor StAR protein expression was altered by the administration of DG and DT (Fig. 8). Under the challenge with ACTH, StAR protein expression were increased 41% compared with its control, and this increased effect was reduced by DG and DT. StAR mRNA expression was decreased in cells treated with DG by 31%, but significantly reduced in ZFR cells treated with DT (69%, $P < 0.05$) or ouabain (62%, $P < 0.05$) (Fig. 9).

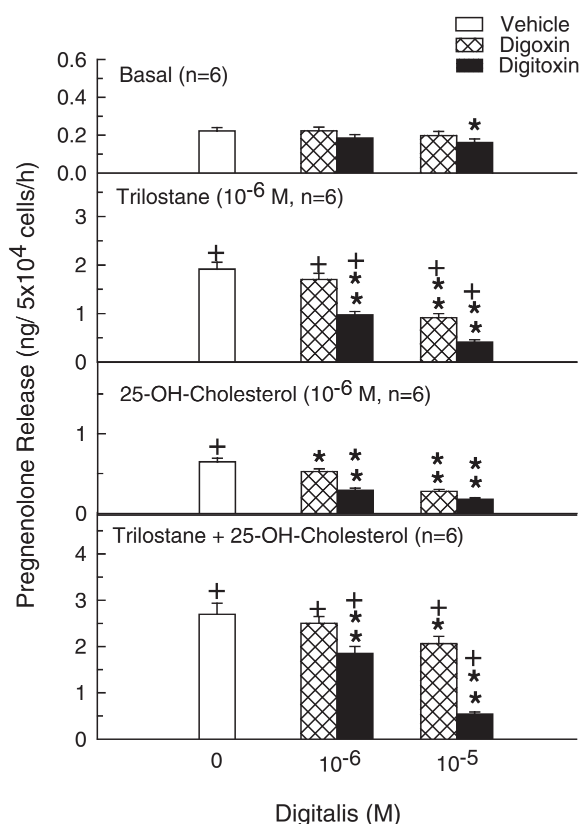


**Fig. 6.** Effects of DG and DT ($10^{-6}$–$10^{-5}$ M) on corticosterone release by ovx rat ZFR cells treated with or without trilostane ($10^{-6}$ M), 25-OH-Cholesterol ($10^{-5}$ M) and trilostane plus 25-OH-Cholesterol. Each column represents mean ± SEM. *, **: $P < 0.05$, $P < 0.01$ compared with corresponding vehicle group. +: $P < 0.01$ compared with basal group.

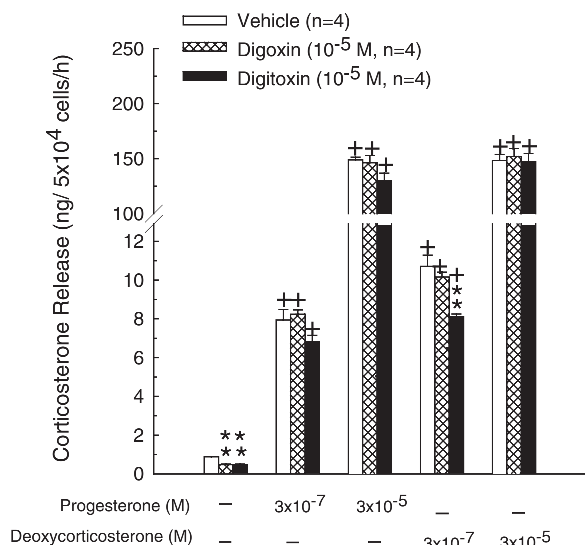


**Fig. 5.** Effects of DG and DT ($10^{-6}$–$10^{-5}$ M) on corticosterone release by ovx rat ZFR cells treated with or without progesterone and deoxycorticosterone ($3 \times 10^{-7}$ or $3 \times 10^{-5}$ M). Each column represents mean ± SEM. *, **: $P < 0.05$, $P < 0.01$ compared with corresponding vehicle group. +: $P < 0.01$ compared with basal group.

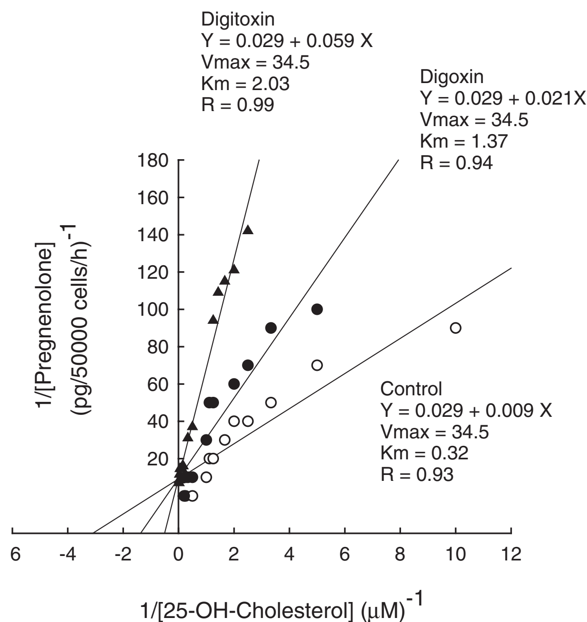


**Fig. 7.** Kinetic analysis of DG and DT ($10^{-5}$ M) inhibition of P450scc function. Double reciprocal plot of data were obtained from cultured ZFR cells challenged with 25-hydroxy-cholesterol ($10^{-7}$ M–$6 \times 10^{-5}$ M). The $V_{max}$ of the both group were almost the same (34.5 ng/h/$1 \times 10^5$ cells). The $K_m$ of the DG-treated group (1.37 μM) and DT-treated group (2.03 μM ) was greater than the control group (0.32 μM).

## DISCUSSION

The present results demonstrate that DG and DT, but not ouabain, inhibit the spontaneous, Ang II- and ACTH-stimulated secretion of corticosterone and decrease the activity of P450scc by acting directly on rat ZFR cells. The expression of StAR mRNA was reduced by DT and ouabain. Furthermore, our data suggest that the inhibitory effects of DG and DT on corticosterone release are mediated distal to the formation of cyclic AMP and intracellular calcium activity by $Na^+$, $K^+$-ATPase-independent pathway. The $V_{max}$ of DG and DT group was the same as the control group, but the $K_m$ was higher than control value. This was a competitive inhibition mechanism, DG and DT might interfered the formation of binding complex of P450scc and cholesterol or inhibit the function of P450scc in situ in the ZFR cells.

It has been reported that administration of DG for 2 years decreases the plasma testosterone and LH level in male patients with cardiac function capacity in late class II and early class III stages [Stoffer et al., 1973; Neri et al., 1987]. In our previously studies, we have demonstrated that DG 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 attenuation of the activities of cytochrome P450scc enzyme and 3β-HSD in rat testicular interstitial cells [Lin et al., 1998; Wang et al., 1999]. Recently, we also found that both DG and DT decrease the pregnenolone release by rat luteal cells [Chen et al., 2002], and progesterone release by rat granulosa cells via a $Na^+$, $K^+$-ATPase-independent pathway, involving the inhibition of post-cyclic AMP pathway and cytochrome P450scc [Chen et al., 2001].

ACTH is the major tropic hormone to regulate steroidogenesis in adrenal cortical cells [Hadley, 2000]. In the present study, we found that the basal and ACTH-induced production of corticosterone by ZFR cells were attenuated by DG and DT, but not by ouabain (Fig. 2), a selective $Na^+$,$K^+$-ATPase inhibitor [Matsumoto et al., 2000]. These result indicate that DG and DT decrease the corticosterone release by ZFR cells via a $Na^+$,$K^+$-ATPase-independent pathway.

It is well established that ACTH stimulates the secretion of corticosterone via mechanisms involving increased production of cyclic AMP. In accordance with these data, we observed a marked increase in the corticosterone secretion of cells exposed to 8-Br-cAMP (Fig. 1) and forskolin (Fig. 2), and are decreased dose-dependently by administration of DG and DT, these data suggest that both the activity of adenylyl cyclase and the function of cyclic AMP are involved in the mechanism of the effects of DG and DT on the production of corticosterone in ZFR cells. In the presence of IBMX (phosphodiesterase inhibitor), administration of forskolin produced a significant approximately 12-fold increase in corticosterone secretion in ZFR cells (Fig. 2). DG and DT also significantly decreased the IBMX plus forskolin-stimulated corticosterone production in a dose-dependently manner, it did not prevent the inhibitory effects of DG and DT. These observations suggest that the inhibitory effects of DG and DT on corticosterone production in rat ZFR cells are associated with a post-cyclic AMP pathway.

In the present study, CPA, an inhibitor of sarcoplasmic reticular $Ca^{2+}$-ATPase [Soler et al., 1998]; A23187, a $Ca^{2+}$ ionophore and NAADP, an activator of intracellular $Ca^{2+}$ release via an $IP_3$ and cyclic ADP ribose-independent mechanism [Lee and Aarhus,

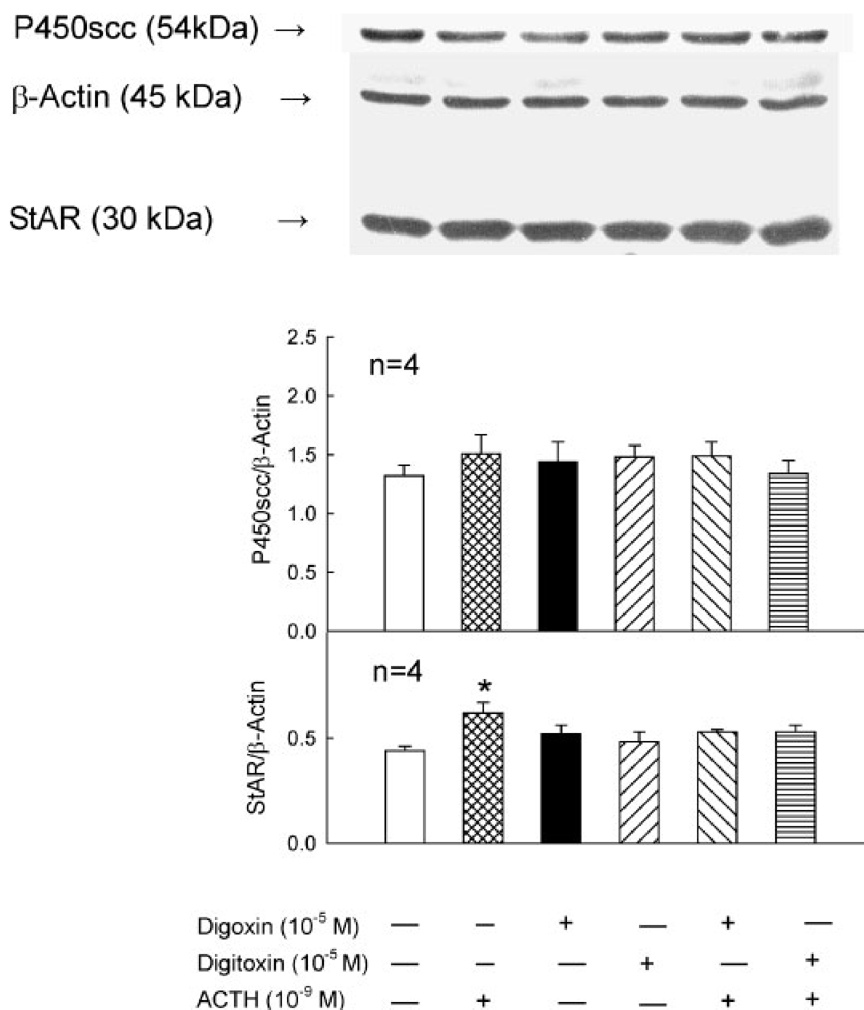


**Fig. 8.** P450scc and StAR protein expression under DG, DT ($10^{-5}$ M), and ACTH ($10^{-9}$ M) treatment for 2 h. Western blot analysis of cell extract subjected to SDS–PAGE and developed by ECL reaction. P450scc (54 kDa) and StAR (30 kDa) were detected by a concomitant with the P450scc and StAR antisera. The level of the StAR protein was enhanced by ACTH, but the P450scc and StAR protein were not affected by the DG and DT ($10^{-5}$ M) treatment. This experiment was repeated four times with similar results. *: $P < 0.05$ as compared with vehicle group.

1995] were used to test the role of $Ca^{2+}$ in the observed DG and DT effects. We found that intracellular $Ca^{2+}$ mobilization and extracellular $Ca^{2+}$ influx were involved in DG and DT action. Our results showed that, CPA, A23187 and NAADP increased corticosterone release by ZFR cells, but DG and DT also inhibit the stimulatory effect. It suggested that the inhibitory effects of DG and DT on corticosterone release in rat ZFR cells were associated with a post intracellular $Ca^{2+}$ mobilization pathway. $Ca^{2+}$-stimulated corticosterone production was reduced by DG and DT, suggesting that DG and DT might block the $Ca^{2+}$-dependent corticosterone biosynthesis pathway.

It is well known that the first or rate-limiting step of steroidogenesis is the conversion of cholesterol into pregnenolone by P450 side-chain cleavage (P450scc) enzyme. In the present study, trilostane was employed to block 3β-HSD activity, since the accumulation of pregnenolone is a sensitive index for the activity of P450scc. 3β-HSD is one of the enzymes for glucocorticoid biosynthesis. In order to observe the effects of DG and DT on the activity of P450scc, rat ZFR cells were incubated with or without trilostane (3β-HSD blocker) and/or 25-OH-Cholesterol in the presence or absence of DG and DT. Our results showed that despite of the presence of 25-OH-Cholesterol, administration of ZFR cells with trilostane increased the production of pregnenolone. DG at $10^{-5}$ M also decreased the production of pregnenolone in response to trilostane in combination with or

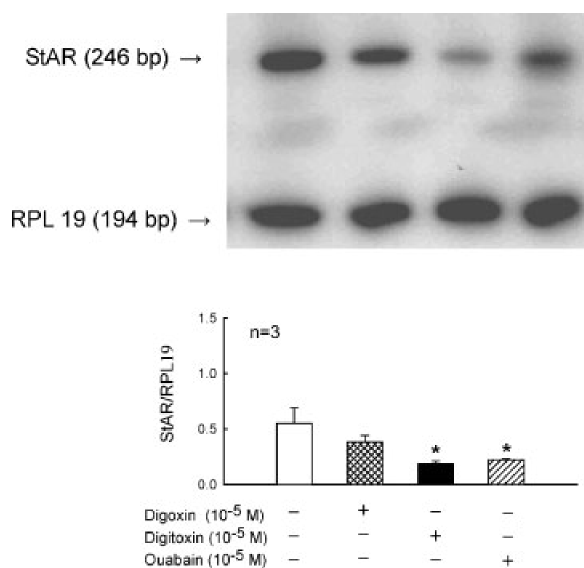


**Fig. 9.** DT and ouabain ($10^{-5}$ M) diminished StAR mRNA expression in ZFR cells. After rat ZFR cells was challenged by DG, DT and ouabain for 30 min, RNA was extracted and 100 ng RNA were assayed by relative-quantitative RT-PCR. Ribosomal protein L19 served as an internal control. The autoradiogram results are shown in the bottom panels. *: $P < 0.05$ as compared with vehicle group.

without 25-OH-Cholesterol. These results suggest that DG and DT reduced the secretion of corticosterone by ZFR cells partially via an inhibition on the activity of P450scc, the rate-limiting enzyme for the conversion of cholesterol to pregnenolone during corticosterone biosynthesis [Hadley, 2000]. Certainly, DG and DT reduced markedly the activity of this enzyme but fail to influence the activity of other steroidogenic enzymes tested.

The final step in corticosterone biosynthesis is the conversion of deoxycorticosterone to corticosterone under the catalyzation of the enzyme 11β-hydroxylase. We found that administration of high dose of DOC ($3 \times 10^{-5}$ M) could fully abolish the inhibitory effect of DG and DT on corticosterone secretion (Fig. 5). These data suggest that the inhibition of corticosterone secretion by DG and DT was not related to the activity of 11β-hydroxylase.

In order to investigate the mechanism by which DG and DT inhibit P450scc function, we challenged ZFR cells with serial doses of 25-OH-Cholesterol. The kinetic analysis of 25-OH-Cholesterol-treated ZFR cells revealed that P450scc has an apparent $K_m$ of 0.32 μM and a $V_{max}$ of 34.5 ng/h/5 × $10^4$ cells. The $V_{max}$ of the DG and DT group was the same as the control group, but the $K_m$ was 1.37 and 2.03 μM, respectively (Fig. 7). This was consistent with a competitive inhibition mechanism, and indicates that DG and DT might interfere with the formation of the binding complex of P450scc and cholesterol, or inhibit the function of P450scc in situ in ZFR cells.

The rate-limiting step in corticosterone biosynthesis is conversion of cholesterol to pregnenolone by P450scc, and there is another important enzyme identified as involved in the acute regulation of steroid production in steroidogenic tissue, StAR. The StAR protein represents a most attractive candidate for the transfer of cholesterol from cellular stores to the inner mitochondrial membrane. Based on data obtained from the regulated expression of StAR and the observed accompanying increased in steroid biosynthesis [Clark et al., 1994; Stocco and Clark, 1996], it is proposed that StAR is rapidly synthesized in the cytosol in response to hormone stimulation and is quickly targeted to the mitochondria via a specific receptor on the mitochondria outer membrane. After transferring cholesterol into mitochondria, StAR would be catabolized. In the present studies, the protein and mRNA expression of P450scc and StAR were examined by Western blot and a semiquantitative RT-PCR assay. Figure 9 show that relative quantitation is indeed feasible for each individual expression because the housekeeping reference gene of choice, the ribosomal protein L19 [Chan et al., 1987; Camp et al., 1991] could be used as a reference gene for the investigation of StAR and P450scc expression [Ronen-Fuhrmann et al., 1998]. Under the challenge of DT but not DG, StAR mRNA expression was rapidly inhibited in 30 min, which suggested that DT may regulate StAR mRNA expression rapidly.

Under acute ACTH stimulation, the level of StAR mRNA was increased within 0.5 h in ZFR cells [Lehoux et al., 1998]. Increases in the levels of StAR protein were also found in ZFR cells, but were delayed compared with those of their mRNA [Lehoux et al., 1998]. Few changes were observed in the P450scc protein content [Lehoux et al., 1998]. Based on our Western blot data, there was no significant difference observed in StAR and P450scc protein expression between the digitalis group and the control group after incubation of ZFR cells with digitalis for 2 h (Fig. 8). But under the challenge with ACTH, StAR protein expression was significantly increased by 41%, compared with control

groups, and this stimulatory effect was reduced by DG and DT (Fig. 8). Since our results indicated that DG and DT diminished corticosterone production, it suggests that the acute inhibitory effects of DG and DT on corticosterone production may be an inhibition of P450scc function and StAR protein expression. In our studies, although ZFR cells challenged by ACTH only 2 h, we found that ACTH-enhanced StAR protein expression was reduced by DG and DT to the basal level. This result indicate that DG and DT inhibit corticosterone secretion partially through suppression of StAR protein expression. Inasmuch as digitalis inhibited StAR expression at the mRNA level and ACTH-induced protein level, we supposed that digitalis may prevent the translocation of StAR to mitochondria which induced catabolism of StAR.

In summary, the present results further demonstrated that DG and DT can act directly on rat ZFR cells via a $Na^{+}$,$K^{+}$-ATPase-independent mechanism involving the inhibition of the activities of adenylyl cyclase, intracellular calcium, and P450scc enzyme as well as the reduction of StAR mRNA expression.

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