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Inhibitory effects of digoxin and digitoxin on corticosterone production in rat zona fasciculata-reticularis cells

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1 The aim of the present study was to investigate the direct effects and action mechanisms of digitalis on the production of corticosterone in rat adrenocortical cells.

2 Male rats were challenged with digoxin ($1\,\mu g\,ml^{-1}\,kg^{-1}$) in the presence or absence of adrenocorticotropin (ACTH, $5\,\mu g\,ml^{-1}\,kg^{-1}$) administered by intravenous injection to the right jugular vein. Blood samples were collected at 0, 30, 60, and 120 min following the challenge. The concentration of corticosterone in the rat plasma samples was measured by radioimmunoassay.

3 Zona fasciculata-reticularis (ZFR) cells in male rats were prepared and then incubated with or without digoxin or digitoxin in the presence or absence of ACTH (10^{-9} M), forskolin (10^{-7} M), 8-bromo-cyclic 3′ : 5′-adenosine monophosphate (10^{-4} M), cyclopiazonic acid (CPA, 10^{-5} M), trilostane (10^{-6} M), 25-OH-cholesterol (10^{-5} M), pregnenolone (10^{-5} M), progesterone (10^{-5} M), or deoxycorticosterone (10^{-5} M) at 37°C for 1 h before collection of the media. Corticosterone or pregnenolone levels were measured by radioimmunoassay.

4 A single injection of digoxin did not alter the basal level of plasma corticosterone, but did inhibit the level of plasma corticosterone released in response to ACTH *in vivo*.

5 Administration of digoxin or digitoxin decreased both spontaneous and ACTH-stimulated release of corticosterone *in vitro*.

6 Digoxin (10^{-7}–10^{-5} M) and digitoxin (10^{-7}–10^{-5} M), but not ouabain (10^{-7}–10^{-5} M), dose-dependently inhibited corticosterone production in response to forskolin and 8-Br-cyclic AMP in rat ZFR cells.

7 Both digoxin (10^{-6}–10^{-5} M) and digitoxin (10^{-6}–10^{-5} M) attenuated corticosterone production in response to CPA.

8 Digoxin (10^{-5} M) or digitoxin (10^{-5} M) inhibited cytochrome *P*450 side-chain cleavage enzyme (cytochrome *P*450scc) activity (catalyses conversion of cholesterol to pregnenolone in the presence of trilostane) in rat ZFR cells.

9 The enzyme activity of 11 β-hydroxylase (catalyses conversion of deoxycorticosterone to corticosterone) in ZFR cells was also inhibited by the administration of digoxin (10^{-5} M) or digitoxin (10^{-5} M).

10 These results together suggest that digoxin and digitoxin decrease the release of corticosterone by acting directly on ZFR cells *via* a Na^+, K^+-ATPase-independent mechanism involving the inhibition of the activities of adenylyl cyclase, cytochrome *P*450scc and 11 β-hydroxylase, as well as the functioning of cyclic AMP and intracellular calcium.

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Abbreviations: ACTH, adrenocorticotropin; 25-OH-cholesterol, 25-hydroxy-cholesterol; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 8-Br-cAMP, 8-bromo-adenosine 3′ : 5′-cyclic monophosphate; BSA, bovine serum albumin; CPA, cyclopiazonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; P450scc, cytochrome P450 side-chain cleavage enzyme; ZFR, zona fasciculata-reticularis

Introduction

Digoxin and digitoxin are two major components of digitalis, which are cardiac glycosides obtained from leaves of the foxglove, *Digitalis purpurea* or *Digitalis lanata* (Smith *et al*., 1984). It is known that these substances produce a profound beneficial effect on failing heart muscle. Indeed, digoxin, digitoxin and related drugs have widespread clinical use in the treatment of heart failure and atrial dysrhythmias (Antman & Smith, 1985). The direct effects of both digitalis and ouabain (a selective Na^+, K^+-ATPase inhibitor, Matsumoto *et al*., 2000) have been attributed to the inhibition of Na^+, K^+-ATPase, an

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enzyme that provides the energy for the active transport of Na^+ and K^+ across cell membranes (Blanco & Mercer, 1998). However, sodium pump inhibition does not seem to be the exclusive mediator of the haemodynamic effects of the cardiac glycosides, since chronic hypertension can be induced in rats by the administration of ouabain but not digoxin or digitoxin (Manunta *et al*., 2000). Furthermore, in rats with ouabain-dependent hypertension, treatment with digoxin or digitoxin normalizes blood pressure even though circulating ouabain levels remain elevated (Manunta *et al*., 2000). The presence of structure-specific mechanisms that regulate the effects of cardiac glycosides on chronic blood pressure was thus suggested.

It has been shown that administered ouabain may act directly on adrenocortical cells to reduce aldosterone secretion (Cushman, 1969; Szalay, 1993), whereas the production of corticosterone is not altered (Cushman, 1969). Although in healthy men treatment with digoxin does not alter the levels of plasma steroids including cortisol and aldosterone (Kley *et al*., 1982), patients receiving long-term digoxin therapy show decreased plasma testosterone and luteinizing hormone (LH) levels (Stoffer *et al*., 1973; Neri *et al*., 1980). This may account for the inhibition of sexual desire and excitement observed in these subjects. Recently, we have found that both digoxin and digitoxin inhibit the production of testosterone through a decrease in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in rat testicular interstitial cells (Lin *et al*., 1998; Wang *et al*., 1999). Meanwhile, both digoxin and digitoxin decrease progesterone release by granulosa cells *via* a Na^+, K^+-ATPase-independent mechanism involving the inhibition of the post-cyclic AMP pathway, cytochrome *P*450 side-chain cleavage enzyme (*P*450scc) and steroidogenic acute regulatory (StAR) protein functions (Chen *et al*., 2001; 2002). It is apparent that the biosynthesis of gonadal steroids is inhibited by digoxin and digitoxin. However, the effects and mechanisms of digitalis on the production of glucocorticoids in adrenocortical cells are still not clear.

Since glucocorticoids are adrenal steroids produced in response to various stressors, it is important to understand if the administration of digitalis affects the biosynthesis of glucocorticoids. In the present study, we found that digoxin inhibited adrenocorticotropin (ACTH)-stimulated release of corticosterone in rat adrenocortical cells both *in vivo* and *in vitro*. These results provide useful information on the regulation of anti-inflammation and glucose metabolism in response to digitalis.

Methods

Animals

Male Sprague–Dawley rats weighing 300–350 g were housed in a temperature-controlled room ($22 \pm 1^\circ C$) with 14 h of artificial illumination daily (06:00–20:00). Food and water were given *ad libitum*.

Effect of digoxin on corticosterone in vivo

Male rats were anaesthetized with ether and then catheterized *via* the right jugular vein (Wang *et al*., 1989; 1994; Tsai *et al*., 1996). After 20 h, the conscious rats were injected intravenously with digoxin ($1\,\mu g\,ml^{-1}\,kg^{-1}$), ACTH ($5\,\mu g\,ml^{-1}\,kg^{-1}$), or ACTH plus digoxin, *via* the jugular catheter. Blood samples (0.3 ml each) were collected at 0, 30, 60 and 120 min after the challenge. Plasma was separated by centrifugation at $10{,}000 \times g$ for 1 min. Each plasma sample was subjected to ether extraction and then the concentration of corticosterone measured by radioimmunoassay (RIA).

Preparation of zona fasciculata-reticularis (ZFR) cells for cell culture

A rat adrenocortical preparation was enriched with ZFR cells for culture following a method described elsewhere by Purdy *et al*. (1991) with minor modifications (Lo *et al*., 1998a, b). After decapitation, the adrenal glands were rapidly excised, cleaned, and then stored in an ice-cold 0.9% NaCl solution. The encapsulated glands were separated into the outer zone (mainly zona glomerulosa) and inner zone (mainly ZFR) fractions using forceps. The inner zone fractions from six to eight adrenal glands were incubated with collagenase ($2\,mg\,ml^{-1}$, Sigma, U.S.A.) at 37°C in a vibrating waterbath (100–110 strokes per minute) for 1 h. The collagenase was dissolved in 2–4 ml of Krebs-Ringer bicarbonate buffer ($3.6\,mmol\ K^+\,l^{-1}$, $11.1\,mmol\,glucose\,l^{-1}$, with 0.2% bovine serum albumin (BSA) medium, KRBGA, pH 7.4). ZFR cells were dispersed by repeated pipetting and filtering through a nylon mesh. After centrifugation at $200 \times g$ for 10 min, the cells were washed in KRBGA medium and centrifuged again. Erythrocytes were separated from ZFR cells by hypotonic shock with 4.5 ml distilled water for a few seconds. The ZFR cells were then mixed with 0.5 ml 10× HBSS, pH 7.4. Following centrifugation at $200 \times g$ for 10 min, the supernatant was discarded and the pellets were resuspended in 3 ml of KRBGA solution. An aliquot (20 μl) was used to count the cells in a haemocytometer after staining with 0.05% nigrasin stain. The viability of isolated cells was 70–75%. Cells in the culture medium were further diluted to a concentration of $5 \times 10^4\,cells\,ml^{-1}$ and aliquotted into the test tubes.

Effects of digoxin and digitoxin on basal and cyclic AMP-related corticosterone secretion

Aliquots (1 ml) of cell suspension ($5 \times 10^4\,cells\,ml^{-1}$) were preincubated with KRBGA medium in polyethylene tubes for 1 h at 37°C in a controlled atmosphere (95% O_2, 5% CO_2), shaken at 100 cycles min^{-1}. The supernatant fluid was decanted after centrifugation of the tubes at $100 \times g$ for 10 min. Resuspended rat ZFR cells ($5 \times 10^4\,cells\,ml^{-1}$) were then incubated for 1 h with digoxin (10^{-8}–10^{-5} M), digitoxin (10^{-8}–10^{-5} M), or ouabain (10^{-8}–10^{-5} M) in the presence of ACTH (10^{-9} M, Sigma, U.S.A.), forskolin (an adenylyl cyclase activator, 10^{-7} M, Sigma, U.S.A.), or 8-Br-cyclic AMP (a membrane-permeable analogue of cyclic AMP, 10^{-4} M, Sigma, U.S.A.). After 1 h incubation, the cells were centrifuged at $200 \times g$ at 4°C for 10 min. The supernatant fluid was stored at −20°C prior to analysis of corticosterone levels by RIA. Forskolin was dissolved initially in dimethylsulphoxide (DMSO, Sigma, U.S.A.) and diluted 1 : 10,000 in medium before use. In all instances, vehicle-treated controls were run in parallel.

Effects of digoxin and digitoxin on corticosterone response to an inhibitor of Ca^{2+}-ATPase

Rat ZFR cell suspensions were pre-incubated for 1 h and were then incubated for a further 1 h with or without digoxin (10^{-5} M) or digitoxin (10^{-5} M) in the presence of cyclopiazonic acid (CPA, a specific inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum, 10^{-5} M, Sigma, U.S.A.). Following incubation, the cells were centrifuged at $200 \times g$ for 10 min and the supernatant fluid stored at -20°C until corticosterone analysis by RIA.

Effects of digoxin and digitoxin on the activities of steroidogenic enzymes

To measure the effect of digitalis on the activity of *P*450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), 21-hydroxylase, and 11β-hydroxylase, ZFR cells were preincubated and then further incubated for 1 h with medium containing 25-hydroxycholesterol (25-OH-cholesterol; 10^{-5} M), pregnenolone (10^{-5} M), progesterone (10^{-5} M), deoxycorticosterone (10^{-5} M), or trilostane (an inhibitor of 3β-HSD, 10^{-6} M). After incubation and centrifugation at $200 \times g$ for 10 min, the supernatant was collected to measure the concentration of corticosterone or pregnenolone by RIA.

RIA of corticosterone and pregnenolone

The concentrations of corticosterone in plasma extract and media were determined by RIA as described elsewhere (Chen *et al*., 1997; Lo *et al*., 1998a, b). With anticorticosterone no. PSW#4–9, the sensitivity of corticosterone detection was 5 pg ml^{-1}. The intra- and inter-assay coefficients of variation were 3.4% ($n=5$) and 9.5% ($n=5$), respectively.

The concentration of pregnenolone in media was determined by RIA as previously described (Kau *et al*., 1999; Chang *et al*., 2002). The anti-pregnenolone antiserum was purchased from Biogenesis, Inc. (Sandown, NH, U.S.A.). The sensitivity of pregnenolone RIA was 16 pg per tube. The intra- and inter-assay coefficients of variation were 2.4% ($n=6$) and 3.8% ($n=4$), respectively.

Materials

BSA, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid (HEPES), Hank's balanced salt solution (HBSS), Medium 199, sodium bicarbonate, penicillin-G, streptomycin, heparin, collagenase, ACTH, forskolin, 8-Br-cyclic AMP, CPA, 25-OH-cholesterol, pregnenolone, progesterone, deoxycorticosterone, corticosterone, ouabain, digitoxin and digoxin were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{3}H]-corticosterone was obtained from Amersham International Plc. (Bucks, U.K.). The doses of drugs are expressed in their final molar concentrations in the assay.

Statistical analysis

All values are given as the mean $\pm$ standard error of the mean (s.e.m.). In some cases, means were tested for homogeneity by a two-way analysis of variance (ANOVA), and the difference between specific means was tested for significance by Duncan's multiple-range test (Steel & Torrie, 1981). In other cases, the Student's *t*-test was employed. A difference between two means was considered statistically significant when $P<0.05$.

Results

Effects of digoxin on plasma corticosterone concentration

The mean basal concentration of plasma corticosterone was 81.75 ± 8.69 ng ml^{-1} (Figure 1). Injection of digoxin (1 μg ml^{-1} kg^{-1}) did not alter the corticosterone levels. Within 30 min following a single injection of ACTH (5 μg ml^{-1} kg^{-1}), the level of plasma corticosterone was increased from 85.34 ± 11.34 to 295.17 ± 15.96 ng ml^{-1} ($n=5$) (Figure 1). However, the peak level of plasma corticosterone released in response to ACTH plus digoxin was 170.03 ± 15.39 ng ml^{-1}, which is 58% of those treated with ACTH only. The levels of plasma corticosterone decreased rapidly from 30 to 120 min after ACTH injection, and were lower following treatment with ACTH plus digoxin than with ACTH treatment alone (98.84 ± 15.39 and 15.03 ± 1.11 ng ml^{-1} *versus* 168.55 ± 22.74 and 44.17 ± 16.77 ng ml^{-1} at 60 and 120 min, $n=5$, respectively).

Effects of digoxin, digitoxin and ouabain on corticosterone production in vitro

The spontaneous release of corticosterone by cultured rat ZFR cells was decreased by the administration of digoxin (10^{-6} and 10^{-5} M), or digitoxin (10^{-7}–10^{-5} M) *in vitro* (0.50 ± 0.09–0.18 ± 0.03 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, *versus* basal level 0.95 ± 0.06 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, $P<0.01$, Figure 2, upper panel). The basal release of corticosterone *in vitro* was not altered by ouabain (10^{-8}–10^{-5} M, 1.21 ± 0.12–1.18 ± 0.16 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$).

Incubation of ZFR cells with ACTH (10^{-9} M) for 1 h resulted in a significant increase in corticosterone secretion

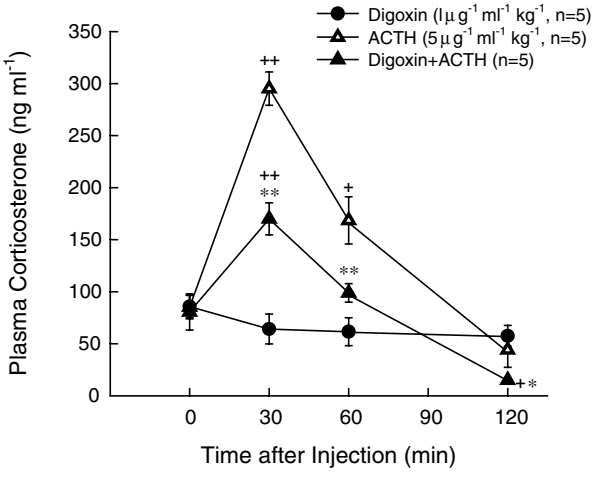

Figure 1 Effects of digoxin on the basal and ACTH-stimulated concentration of plasma corticosterone in male rats. Rats were given a single intravenous injection of digoxin (1 μg ml^{-1} kg^{-1}), ACTH (5 μg ml^{-1} kg^{-1}), or ACTH plus digoxin *via* the right jugular vein. Blood samples were collected *via* the jugular catheter at time intervals following injection. Each value represents mean $\pm$ s.e.m. $^*P<0.05$ and $^{**}P<0.01$ compared with non-digoxin-injected animals at the same time point; $^+P<0.05$ and $^{++}P<0.01$ compared with the value at 0 min.

(ACTH-treated group 37.32±2.00 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, *versus* vehicle group 0.95±0.06 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, $P<0.01$, Figure 2, lower panel). Coincubation of ACTH-treated ZFR cells with digoxin (10^{-8}–10^{-5} M), or digitoxin (10^{-8}–10^{-5} M) dose-dependently decreased the corticosterone release in response to ACTH from 40.36±6.20 to 2.36±0.31 ng, 5×10^4 cells^{-1} h^{-1} ($P<0.05$ or $P<0.01$). None of the doses of ouabain tested reduced the corticosterone released in response to ACTH.

Effects of digoxin, digitoxin and ouabain on adenylyl cyclase activity in ZFR cells

Forskolin (10^{-7} M) stimulated the release of corticosterone by rat ZFR cells (20.83±3.75 ng, 5×10^4 cells^{-1} h^{-1}, $n=7$, *versus* forskolin-free group, 1.30±0.11 ng, 5×10^4 cells^{-1} h^{-1}, $n=7$, $P<0.01$) (Figure 3). Digoxin or digitoxin at 10^{-7}–10^{-5} M significantly decreased forskolin-stimulated release of corticosterone by ZFR cells (digoxin: 11.59±2.42–1.68±0.39 ng 5×10^4 cells^{-1} h^{-1}, $n=7$, $P<0.05$ or $P<0.01$; digitoxin: 10.38±2.35–1.66±0.35 ng, 5×10^4 cells^{-1} h^{-1}, $n=7$, $P<0.01$) (Figure 3). Ouabain at 10^{-7}–10^{-5} M did not alter the stimulatory effects caused by forskolin.

Effects of digoxin, digitoxin and ouabain on cyclic AMP function in ZFR cells

Treatment of ZFR cells with 8-Br-cAMP at 10^{-4} M stimulated the release of corticosterone (49.10±4.04 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$) compared to untreated cells (1.36±0.12 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, $P<0.01$) (Figure 4). Digoxin or digitoxin at 10^{-7}–10^{-5} M dose-dependently reduced the release of corticosterone from ZFR cells by 30–94% (digoxin: 33.31±5.19–11.18±1.20 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, $P<0.05$ or $P<0.01$; digitoxin: 34.34±2.76–2.10±0.16 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, $P<0.01$). Ouabain at 10^{-8}, 10^{-6}, and 10^{-5} M, but not at 10^{-7} M concentration, decreased corticosterone release from ZFR cells by 28–39% (34.72±5.00–29.29±4.06 ng, 5×10^4 cells^{-1} h^{-1}, $n=8$, $P<0.05$ or $P<0.01$) (Figure 4). Administration of digoxin, digitoxin, or ouabain, even at 10^{-5} M, did not have any cytotoxic effects on rat ZFR cells (data not shown).

Effects of digoxin and digitoxin on intracellular calcium activity

CPA is a specific inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum and endoplasmic reticulum that stimulates Ca^{2+} release from intracellular stores by increasing plasma

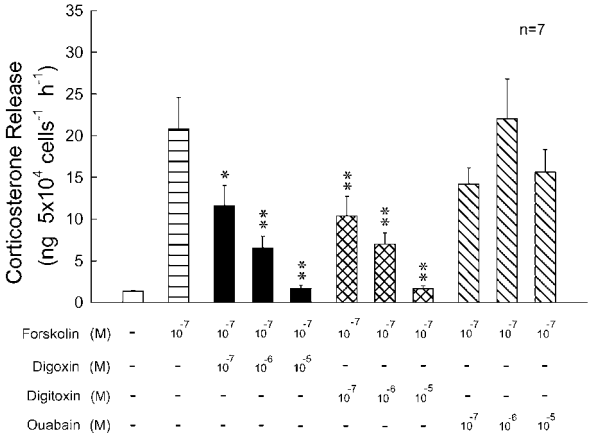

Figure 3 Effects of digoxin, digitoxin and ouabain (10^{-7}–10^{-5} M) on corticosterone release *in vitro* by rat ZFR cells treated with forskolin (10^{-7} M). Each column represents the mean±s.e.m. *$P<0.05$ and **$P<0.01$ compared with forskolin plus vehicle group.

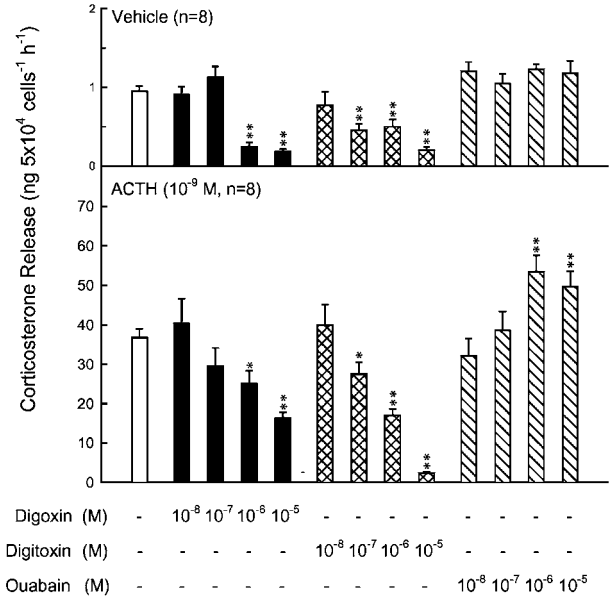

Figure 2 Effects of digoxin, digitoxin and ouabain (10^{-8}–10^{-5} M) on corticosterone release *in vitro* by rat ZFR cells treated with or without ACTH (10^{-9} M). Each column represents mean±s.e.m. *$P<0.05$ and **$P<0.01$ compared with the corresponding control group.

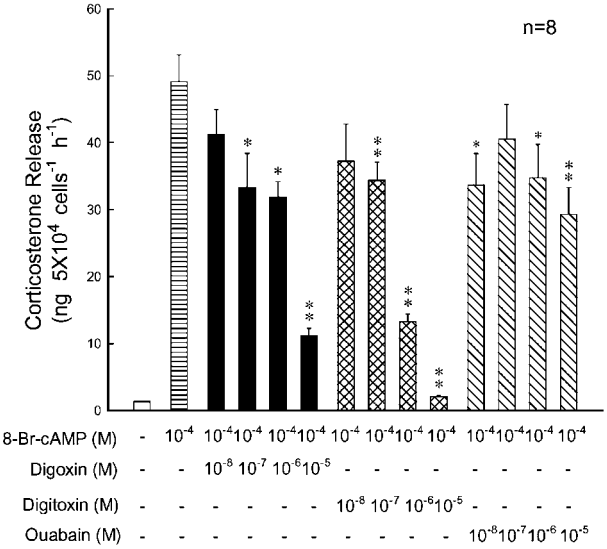

Figure 4 Effects of digoxin, digitoxin and ouabain (10^{-8}–10^{-5} M) on corticosterone release *in vitro* by rat ZFR cells treated with 8-Br-cAMP (10^{-4} M). Each column represents mean±s.e.m. *$P<0.05$ and **$P<0.01$ compared with 8-Br-cAMP group.

membrane Ca^{2+} permeability without increasing intracellular inositol trisphosphate (IP_3) levels (Demaurex *et al*., 1992; Suzuki *et al*., 1992; Uyama *et al*., 1992). Administration of CPA (10^{-5} M) increased corticosterone release by 60% (1.63 ± 0.06 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, *versus* 1.02 ± 0.14 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, $P<0.01$) (Figure 5). Treatment with digoxin or digitoxin at 10^{-6}–10^{-5} M resulted in a dose-dependent inhibition of the production of corticosterone in response to CPA (digoxin 10^{-6}–10^{-5} M, 1.44 ± 0.09 and 0.90 ± 0.09 ng, 5×10^4 cells^{-1} h^{-1}, respectively, $n=6$, $P<0.05$ and $P<0.01$; digitoxin 10^{-6}–10^{-5} M, 1.02 ± 0.03 and 0.61 ± 0.03 ng, 5×10^4 cells^{-1} h^{-1}, respectively, $n=6$, $P<0.01$).

Effects of digoxin and digitoxin on the activities of steroidogenic enzymes

In vitro administration of the steroidogenic precursors 25-OH-cholesterol, pregnenolone, progesterone or deoxycorticosterone, at a concentration of 10^{-5} M, significantly increased corticosterone production in ZFR cells (11.63 ± 2.39 ng, 5×10^4 cells^{-1} h^{-1} in response to 25-OH-cholesterol; 52.61 ± 1.41 ng 5×10^4 cells^{-1} h^{-1} in response to pregnenolone; 72.07 ± 12.17 ng, 5×10^4 cells^{-1} h^{-1} in response to progesterone; and 129.94 ± 6.60 ng, 5×10^4 cells^{-1} h^{-1} in response to deoxycorticosterone, $n=8$, compared to basal levels of 1.34 ± 0.12, 1.75 ± 0.11, 1.89 ± 0.42 and 1.40 ± 0.09 ng, 5×10^4 cells^{-1} h^{-1}, respectively, $n=8$, $P<0.01$) (Figure 6). Coincubation of these precursors with digoxin or digitoxin (10^{-6}–10^{-5} M) resulted in a decrease in corticosterone production (digoxin: 6.64 ± 0.80 ng, 5×10^4 cells^{-1} h^{-1} in response to 25-OH-cholesterol; 33.79 ± 0.82 ng, 5×10^4 cells^{-1} h^{-1} in response to pregnenolone; 20.91 ± 2.50 ng, 5×10^4 cells^{-1} h^{-1} in response to progesterone; 51.57 ± 7.24 ng, 5×10^4 cells^{-1} h^{-1} in response to deoxycorticosterone, $n=8$, $P<0.01$; digitoxin: 1.19 ± 0.53 ng, 5×10^4 cells^{-1} h^{-1} in response to 25-OH-cholesterol; 29.82 ± 1.21 ng, 5×10^4 cells^{-1} h^{-1} in response to pregnenolone; 20.23 ± 1.73 ng, 5×10^4 cells^{-1} h^{-1} in response to progesterone; 59.30 ± 10.65 ng, 5×10^4 cells^{-1} h^{-1} in response to deoxycorticosterone, $n=8$, $P<0.01$).

To further study the changes in the activity of *P*450scc, which converts cholesterol to pregnenolone, an inhibitor of 3β-HSD, trilostane, was used. Since pregnenolone is produced and accumulates in the ZFR cells following 3β-HSD inhibition by trilostane, the change in pregnenolone levels produced by incubation with trilostane may reflect changes in *P*450scc activity. Addition of trilostane, 10^{-5} M, to ZFR cells increased pregnenolone release (1.87 ± 0.23 ng, 5×10^4 cells^{-1} h^{-1} compared to control 0.14 ± 0.02 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, $P<0.01$) as did treatment with 25-OH-cholesterol (10^{-6} M) plus trilostane (2.15 ± 0.15 ng, 5×10^4 cells^{-1} h^{-1} *versus* control 0.46 ± 0.04 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, $P<0.01$) (Figure 7). Administration of digoxin (10^{-5} M) or digitoxin (10^{-5} M) markedly decreased pregnenolone production in response to vehicle, trilostane, 25-OH-cholesterol and to 25-OH-cholesterol plus trilostane (Figure 7). In detail, response to vehicle (digoxin: 0.09 ± 0.01 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, digitoxin: 0.02 ± 0.01 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, *versus* 0.14 ± 0.02 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, $P<0.05$ or $P<0.01$), to trilostane (digoxin: 0.72 ± 0.07 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, digitoxin:

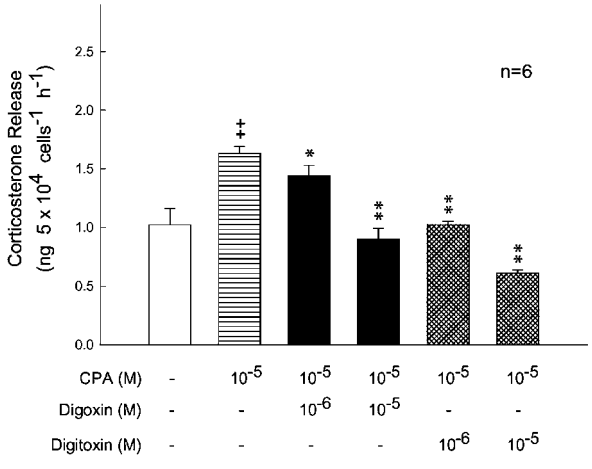

Figure 5 Effects of digoxin and digitoxin (10^{-5} M) on corticosterone release *in vitro* by rat ZFR cells treated with CPA (10^{-5} M). The control group was treated with vehicle only. Each column represents mean $\pm$ s.e.m. $^{++}P<0.01$ compared with the control group, $^{*}P<0.05$ and $^{**}P<0.01$ compared with the CPA group.

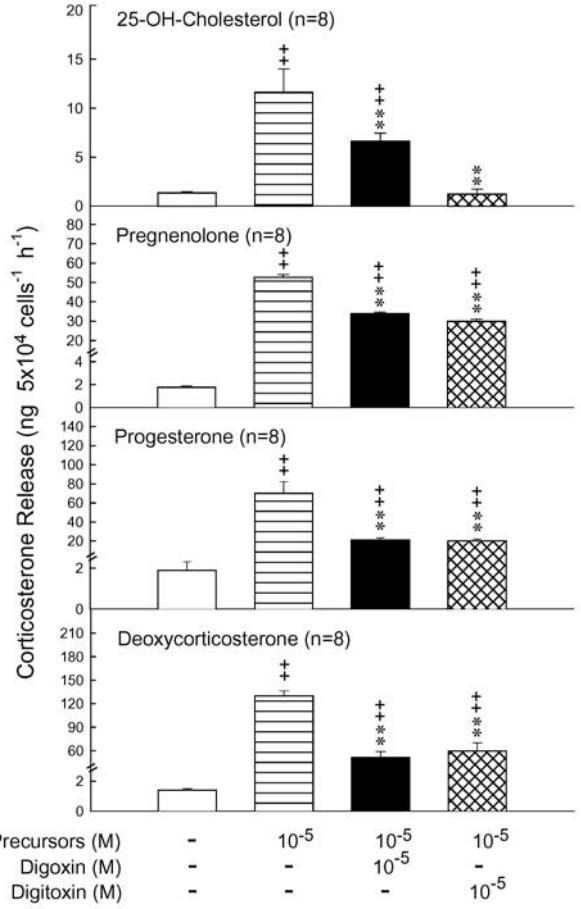

Figure 6 Effects of digoxin and digitoxin (10^{-5} M) on corticosterone release *in vitro* by rat ZFR cells treated with the steroidogenic precursors 25-OH-cholesterol, pregnenolone, progesterone and deoxycorticosterone 10^{-5} M. The control group was treated with vehicle only. Each column represents mean $\pm$ s.e.m. $^{++}P<0.01$ compared with the control group, $^{**}P<0.01$ compared with the corresponding precursor group.

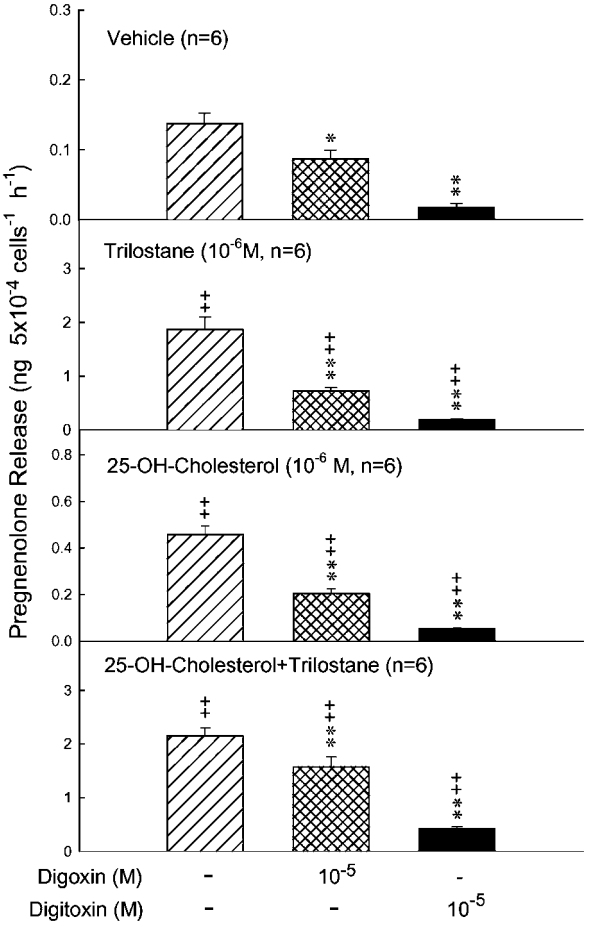

Figure 7 Effects of digoxin and digitoxin (10^{-5} M) on pregnenolone production *in vitro* by rat ZFR cells treated with trilostane and 25-OH-cholesterol. Each column represents the mean ± s.e.m. $^{++}$ $P<0.01$ compared with the vehicle group. $^{*}P<0.05$ and $^{**}P<0.01$ with corresponding control group.

0.18 ± 0.02 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, *versus* 1.87 ± 0.23 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, $P<0.01$), to 25-OH-cholesterol (digoxin: 0.21 ± 0.02 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, digitoxin: 0.05 ± 0.01 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, *versus* 0.46 ± 0.04 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, $P<0.01$) and to 25-OH-cholesterol plus trilostane (digoxin: 1.57 ± 0.18 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, digitoxin: 0.42 ± 0.04 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, *versus* 2.15 ± 0.15 ng, 5×10^4 cells^{-1} h^{-1}, $n=6$, $P<0.05$ or $P<0.01$).

Discussion

The present study examines the effects of digoxin and digitoxin on adrenal function both *in vivo* and *in vitro*. Our results demonstrate that (1) digoxin inhibited the level of plasma corticosterone produced in response to ACTH, (2) digoxin and digitoxin inhibit both the spontaneous and the ACTH-stimulated release of corticosterone *in vitro*, (3) digoxin and digitoxin, but not ouabain, inhibit the effects of cyclic AMP by acting directly on rat ZFR cells, and (4) digoxin and digitoxin inhibit intracellular calcium activity and the activities of steroidogenic enzymes, especially *P*450scc and 11β-hydroxylase in rat ZFR cells.

It has been reported that treatment with digoxin over 2 years reduces the concentration of plasma testosterone and luteinizing hormone in male heart patients with cardiac function capacity in late class II and early class III stages (Stoffer *et al*., 1973; Neri *et al*., 1987). We previously demonstrated that digoxin and digitoxin inhibit the production of testosterone both *in vivo* and *in vitro* through mechanisms involving: (1) decreasing basal and human chorionic gonadotropin (hCG)-stimulated testosterone release, (2) an inefficiency of post-cAMP events and (3) an attenuation of the activity of cytochrome *P*450scc enzyme in rat testicular interstitial cells (Lin *et al*., 1998; Wang *et al*., 1999). Recently, we found that digoxin and digitoxin decrease progesterone release by rat granulosa cells *via* a Na^+, K^+-ATPase-independent mechanism that involves inhibition of the post-cyclic AMP pathway and cytochrome *P*450scc (Chen *et al*., 2001). Based on these observations, it is apparent that digoxin and digitoxin inhibit the production of steroid hormones by acting directly on the gonadal cells *via* a Na^+, K^+-ATPase-independent mechanism involving the post-cAMP pathway and various enzyme activities during steroidogenesis.

It has been well established that ACTH is the major tropic hormone that regulates steroidogenesis in adrenal cortical cells (Hadley, 2000). In the present study, we found that both basal and ACTH-stimulated production of corticosterone by ZFR cells is diminished by digoxin and digitoxin, but not by ouabain (Figure 2), a selective Na^+,K^+-ATPase inhibitor (Matsumoto *et al*., 2000). These data indicate that digoxin and digitoxin decrease the corticosterone release by ZFR cells *via* a Na^+,K^+-ATPase-independent pathway.

Since the stimulatory effects of forskolin and 8-Br-cyclic AMP were decreased dose-dependently by the administration of digoxin and digitoxin, but not by ouabain (Figure 3), we suggest that both the activity of adenylyl cyclase and the function of cyclic AMP are factors in the mechanism of digoxin and digitoxin action on corticosterone production in ZFR cells.

It is well known that digitalis has a positive inotropic effect on the heart. Digitalis cardiac glycosides bind to and partially inhibit Na^+, K^+-ATPase. A chain of events is initiated that ultimately increases calcium availability to contractile elements, thus enhancing myocardial contraction force. Most studies have shown that the positive inotropic effects of digitalis glycosides result from alteration of the mechanism of excitation–contraction coupling. The calcium ion has been shown to be the major trigger that allows coupling of the electrical depolarization of heart-muscle cells to the mechanical contractile event (Smith, 1973; 1988; Smith *et al*., 1984). Since CPA is a specific inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum and endoplasmic reticulum, it stimulates Ca^{2+} release from intracellular stores by increasing plasma membrane Ca^{2+} permeability without increasing intracellular inositol trisphosphate levels (Demaurex *et al*., 1992; Suzuki *et al*., 1992; Uyama *et al*., 1992). Although a change in intracellular calcium was not detected in the present study, the reduction of CPA-induced levels of corticosterone production caused by digoxin and digitoxin treatment indicated the possibility of inhibition occurring at the level of intracellular calcium regulation in ZFR cells.

It is well known that the first, or rate-limiting step, of steroidogenesis is the conversion of cholesterol into pregnenolone by *P*450scc enzyme (Hadley, 2000). 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 3β-HSD. During incubation of ZFR cells with trilostane, either with or without cholesterol, the administration of digitalis decreased the production of pregnenolone by these cells (Figure 7). Apparently, *P*450scc enzyme activity was inhibited by digitalis. Since treatment with digitoxin resulted in a greater inhibition as compared with that caused by treatment with digoxin, digitoxin possessed a greater potency to inhibit *P*450scc enzyme activity. The inhibition of the conversion of corticosterone from deoxycorticosterone by digoxin and digitoxin (Figure 6) suggests that 11β-hydroxylase was inhibited by digitalis. These results indicate that digoxin and digitoxin inhibited the conversion of pregnenolone and progesterone to corticosterone and this implies that the activities of 3β-HSD and 11β-hydroxylase might be inhibited by digitalis. Digoxin and digitoxin were equally potent at inhibiting these enzymes.

In summary, the present results demonstrate that both digoxin and digitoxin inhibit corticosterone production by acting directly on rat ZFR cells. Since ouabain even at the same effective doses of digoxin and digitoxin failed to affect corticosterone production, we suggest that the inhibition of digitalis on corticosterone production might be independent of the action of Na^+, K^+-ATPase, although the present study did not prove that the production of corticosterone could not be inhibited by ouabain at higher doses (e.g. 10^{-4} M). Taken together, the present results suggest that digoxin and digitoxin decrease corticosterone production by ZFR cells *via* a Na^+, K^+-ATPase-independent mechanism involving the inhibition of the activities of adenylyl cyclase, *P*450scc and 11β-hydroxylase, as well as the functioning of cyclic AMP and intracellular calcium.

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