British Journal of Pharmacology (1998) 125, 1635–1640
http://www.stockton-press.co.uk/bjp

Inhibitory effect of digoxin on testosterone secretion through mechanisms involving decreases of cyclic AMP production and cytochrome $P450_{scc}$ activity in rat testicular interstitial cells

[1]Ho Lin, [4]Shyi-Wu Wang, [1]Shiow-Chwen Tsai, [1]Jiann-Jong Chen, [1]Yu-Chung Chiao, [1]Chien-Chen Lu, [2]William Ji-Sien Huang, [3]Guei-Jane Wang, [3]Chieh-Fu Chen & [1,3,5]Paulus S. Wang

[1]Department of Physiology, School of Life Science, and [2]Institute of Clinical Research, School of Medicine, National Yang-Ming University; [3]National Research Institute of Chinese Medicine, Taipei, and [4]Department of Physiology, Chang Gung University, Taoyuan, Taiwan, Republic of China

1 *In vivo* and *in vitro* experiments were performed to examine inhibitory effects of digoxin on testosterone secretion and to determine possible underlying mechanisms.

2 A single intravenous injection of digoxin (1 μg kg^{-1}) decreased the basal and human chorionic gonadotropin (hCG)-stimulated plasma testosterone concentrations in adult male rats.

3 Digoxin (10^{-7}–10^{-4} M) decreased the basal and hCG-stimulated release of testosterone from rat testicular interstitial cells *in vitro*.

4 Digoxin (10^{-7}–10^{-4} M) also diminished the basal and hCG-stimulated production of cyclic 3′:5′-adenosine monophosphate (AMP) and attenuated the stimulatory effects of forskolin and 8-Br-cyclic AMP on testosterone production by rat testicular interstitial cells.

5 Digoxin (10^{-4} M) inhibited cytochrome P450 side chain cleavage enzyme (cytochrome $P450_{scc}$) activity (conversion of 25-hydroxy cholesterol to pregnenolone) in the testicular interstitial cells but did not influence the activity of other steroidogenic enzymes.

6 These results suggest that digoxin inhibits the production of testosterone in rat testicular interstitial cells, at least in part, *via* attenuation of the activities of adenylyl cyclase and cytochrome $P450_{scc}$.

Keywords: Digoxin; testosterone; testicular interstitial cells; cyclic AMP; adenylyl cyclase; cytochrome $P450_{scc}$

Introduction

Digoxin, a purified digitalis preparation, is a cardiac glycosides derived from the flowering plant *Digitalis lanata* (foxglove). It has been known for over 250 years that this substance produces a profound beneficial effect on failing heart muscle and indeed, digoxin and related drugs have found widespread clinical use in the treatment of heart failure and atrial dysrhythmias. The direct positive inotropic actions of digoxin have been attributed to inhibition of Na^+-K^+-ATPase, an enzyme system that provides the energy for active transport of Na^+ and K^+ across the cell membrane. The primary sexual problems reported in male patients suffering from cardiovascular disease are the decrease of sexual desire and excitement phase (Neri *et al*., 1987). Studies of patients receiving long-term digoxin therapy have detected changes in plasma testosterone and luteinizing hormone (LH) (Stoffer *et al*., 1973; Neri *et al*., 1987). The inhibition of sexual desire and excitement might be attributed to relative changes in blood hormones levels following long-term administration of digoxin, but the mechanisms of digoxin effects are not established yet.

The testis has long been known to be the source of testosterone which is responsible for maintenance of spermatogenesis and secondary sexual characteristics in the male. In most species the testis comprises two separate compartments: (1) the seminiferous tubules which contain the Sertoli cells, the peritubular cells and the germ cells and (2) the interstitial compartment which contains the Leydig cells, macrophages, lymphocytes, granulocytes and the cells composing the blood, nerve and lymphatic structures. The biosynthesis of steroid hormones by Leydig cells requires the sequential actions, that convert cholesterol into various steroid classes (Payne & O'Shaughnessy, 1996). Cytochrome P450 side chain cleavage enzyme (cytochrome $P450_{scc}$) is a mitochondrial enzyme which catalyzes the first side chain cleavage of cholesterol to yield pregnenolone. The synthesis of testosterone requires the action of the microsomal enzyme 17α-hydroxylase/$C_{17}-C_{20}$ lyase (cytochrome $P450_c17$) which proceeds in two steps, 17α-hydroxylation and cleavage of the C17–20 bond to yield the C19 steroid dehydroepiandrosterone (DHEA) or androstenedione. 3β-hydroxysteroid dehydrogenase (3β-HSD) catalyzes 3β-hydroxy-5-ene steroids into 3-keto-4-ene steroids (i.e. pregnenolone→progesterone, 17α-hydroxypregnenolone→17α-hydroxyprogesterone, DHEA→androstenedione). The interconversion of androstenedione to testosterone is catalyzed by microsomal enzyme 17-ketosteroid reductase (17-KSR). The intra-testicular mechanism by which digoxin modulates steroidogenesis has not been well-defined, but a number of *in vitro* studies have shown that many compounds (e.g. lead or aminoglutethimide) may directly or indirectly target the enzymes required for the biosynthesis of testosterone in Leydig cells, including cytochrome $P450_{scc}$, $P450_c17$, 3β-HSD, and 17-KSR (Payne & Sha, 1991; Thoreux *et al*., 1995).

The present study was first carried out to examine the effect of digoxin on the basal and human chorionic gonadotropin (hCG)-stimulated secretion of testosterone both *in vivo* and *in vitro* in male rats. The influence of the drug as the hCG-stimulated production of cyclic 3′:5′-adenosine monophosphate (cyclic AMP) and on the activities of the enzymes

[5]Author for correspondence at: Department of Physiology, National Yang-Ming University, Shih-Pai, Taipei, Taiwan, Republic of China.

required for steroidogenesis were also examined. We found that digoxin inhibited testosterone production at least through the mechanisms involving decreases of both cyclic AMP production and cytochrome $P450_{scc}$ activity in rat testicular interstitial cells.

Methods

Animals

Male rats of the Sprague-Dawley strain weighing 300–350 g were housed in a temperature controlled room (22±1°C) with 14 h of artificial illumination daily (06 h 00 min–20 h 00 min) and given food and water *ad libitum*.

In vivo *experiment*

Male rats were anaesthetized with ether and then catheterized *via* the right jugular vein (Wang *et al*., 1989, 1994; Tsai *et al*., 1996a). Twenty hours later, the conscious rats were injected intravenously with vehicle (saline, 1 ml kg^{-1}), hCG (5 iu ml^{-1} kg^{-1}), digoxin (1 μg ml^{-1} kg^{-1}), or hCG plus digoxin, *via* the jugular catheter. Blood samples (0.5 ml each) were collected at 0, 30, 60, 120, 180, and 1440 min after the challenge. An equal volume of saline containing rat red blood cells (45%, v/v) harvested from the donor was injected immediately after each bleeding (Sheu *et al*., 1987). Plasma was separated by centrifugation at 10,000 × g for 1 min. The concentration of testosterone in each plasma sample was measured by radioimmunoassay (RIA) after ether extraction. Preliminary studies demonstrated that the dose of hCG selected produces a submaximal rise in plasma testosterone concentration. Meanwhile, the dose of digoxin employed corresponds with that used clinically.

Preparation of testicular interstitial cells

The method of collagenase dispersion of testicular interstitial cells followed the procedure described by Tsai *et al*., (1997). Five decapsulated testes were added to a 50 ml polypropylene tube containing 5 ml preincubation medium and 700 μg collagenase (Type IA, Sigma, U.S.A.). Preincubation medium were made up of 1% bovine serum albumin (BSA, Fraction V, Sigma, U.S.A.) in Hank's balanced salt solution (HBSS), with N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES, 25 mM), salt bicarbonate 0.35 g l^{-1}, penicillin-G 100 iu ml^{-1}, streptomycin sulphate 50 μg ml^{-1}, heparine 2550 USP K units l^{-1}, pH 7.3, and aerated with 95% O_2 and 5% CO_2. The tube was laid horizontally in a 34°C water bath, parallel to the direction of the shaking. Fifteen minutes after shaking at 100 cycles min^{-1}, the digestion was stopped by adding 35 ml of cold preincubation medium and inverting the tube several times. The tubes were allowed to stand for 5 min and the digest was then filtered through a four-layer fine nylon mesh. Cells were collected by centrifugation at 4°C, 100 × g for 10 min. The cell pellets were washed with deionized water to disrupt red blood cells (RBCs) and the osmolarity was recovered immediately with 10 fold HBSS. Hypotonic shock was repeated twice for disrupting RBCs and cell pellets were resuspended in preincubation medium (substitution of HBSS in preincubation medium with Medium 199, and sodium bicarbonate 2.2 g l^{-1}). Cell concentration (1.0×10^6 cells ml^{-1}), viability (over 97%), and the number of sperm cell (less than 5%) were determined using a haemocytometer and the Trypan blue method. The total cell proteins were determined by the method of Lowry *et al*. (1951). To measure the abundance of Leydig cells in our preparation, the 3β-hydroxysteroid dehydrogenase (3β-HSD) staining method was used (Dirami *et al*., 1991; Krummen *et al*., 1994). The cells (1.0×10^6 cells ml^{-1}) were incubated with a solution containing 0.2 mg ml^{-1} nitro blue tetrozolium (Sigma, U.S.A.), 0.12 mg ml^{-1} 5-androstane-3β-ol-one (Sigma, U.S.A.), and 1 mg ml^{-1} NAD^+ (Sigma, U.S.A.) in 0.05 M PBS, pH 7.4 at 34°C for 90 min. Upon development of the blue formazan deposit sites of 3β-HSD activity, the abundance of Leydig cells was determined by use of a haemocytometer. Our preparation was found to contain approximately 18±2% Leydig cells.

Effects of digoxin on testosterone and cyclic AMP production

Aliquots (1 ml) of cell suspensions (1.0×10^6 cells ml^{-1}) were preincubated with incubation medium in polyethylene tubes for 1 h at 34°C under a controlled atmosphere (95% CO_2 and 5% O_2), shaken at 100 cycles min^{-1}. The supernatant fluid was decanted after centrifugation of the tubes at 100 × g for 10 min. For studying the accumulation of cyclic AMP in response to digoxin, aliquots (1 ml) of cell suspensions (1.0×10^6 cells ml^{-1}) were primed for 30 min with 1 mM 3-isobutyl-1-methylxanthine (IBMX, phosphodiesterase inhibitor, Sigma, U.S.A.). Digoxin (10^{-7}–10^{-4} M), ouabain (10^{-7}–10^{-4} M), hCG (0.05 iu ml^{-1}), hCG plus digoxin or hCG plus ouabain in 200 μl fresh medium in the presence or absence of IBMX was then added to the tubes. After 1 h of incubation, 2 ml ice-cold PBSG buffer (0.1 % gelatin in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.5) was added to stop the incubation. The spent medium was centrifuged at 100 × g and stored at −20°C until analysed for testosterone by RIA. In the presence of IBMX, the cell pellets were mixed with 1 ml of 65% ice-cold ethanol, homogenized by polytron (PT3000, Kinematica Ag., Luzern, Switzerland), and centrifuged at 1500 × g for 15 min. The supernatant fluid was lyophilized in a vacuum concentrator (Speed Vac, Savant, Holbrook, NY, U.S.A.) and stored at −20°C until analysed for cyclic AMP by RIA.

Effects of digoxin on cyclic AMP-related testosterone secretion

Cell suspensions were preincubated for 1 h and then incubated for 1 h with digoxin in the presence of forskolin (an adenylyl cyclase activator, 10^{-6} M, Sigma, U.S.A.) (1 h preincubation including 30 min preincubation and 30 min priming with forskolin at 10^{-6} M) or 8-Br-cyclic AMP (a membrane-permeable analogue of cyclic AMP, 10^{-4} M, Sigma, U.S.A.). At the end of the incubation, 2 ml ice-cold PBSG buffer were added and immediately followed by centrifugation at 100 × g for 10 min at 4°C. The supernatant fluid was stored at −20°C until analysed for testosterone by RIA. Forskolin was dissolved initially in 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 on the biosynthesis pathway of testosterone

Cell suspensions were preincubated for 1 h and then were incubated for 1 h with or without digoxin at 10^{-4} M in the presence or absence of five steroidal precursors. These precursors included 25-hydroxy-cholesterol (membrane-permeable cholesterol, 25-OH-C), pregnenolone (Δ_5P), pro-

gesterone (P), 17α-hydroxy-progesterone (17α-OH-P), and androstenedione (Δ_4). At the end of the incubation, 2 ml ice-cold PBSG buffer were added and immediately followed by centrifugation at $100 \times g$ for 10 min at 4°C. The supernatant fluid was stored at −20°C until analysed for testosterone by RIA.

RIA of testosterone and cyclic AMP

The concentrations of testosterone in extracted samples (recovery 60–65%) were determined by RIA as described previously (Wang *et al*., 1994; Tsai *et al*., 1996a). With anti-testosterone serum No. W8, the sensitivity of testosterone RIA was 2 pg per assay tube. The intra- and interassay coefficients of variation (CV) were 4.1% ($n=6$) and 4.7% ($n=10$), respectively. Standard curves and quality controls were run in duplicate but, due to limited sample, tests were measured by single estimates only.

The concentrations of cyclic AMP were determined by RIA as described elsewhere (Tsai *et al*., 1996b; Lu *et al*., 1996). With anti-cyclic AMP serum No. CV-27 pool, the sensitivity of cyclic AMP was 2 fmol per assay tube. The intra- and interassay coefficients of variation were 6.9% ($n=5$) and 11.9% ($n=5$), respectively. Standard curves and quality controls were run in triplicate but again due to limited material, only single measurement were made on the samples.

Materials

Bovine serum albumin (BSA), N-2-hydroxyethylpiperazine-N′-2-ethane-sulphonic acid (HEPES), Hank's balanced salt solution (HBSS), Medium 199, sodium bicarbonate, penicillin-G, streptomycin, heparine, collagenase, human chorionic gonadotropin (hCG), 3-isobutyl-1-methylxanthine (IBMX), forskolin, 8-Br-cyclic AMP, 25-hydroxy-cholesterol, pregnenolone, progesterone, 17α-hydroxyprogesterone, androstenedione, ouabain, and digoxin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]-testosterone, and ^{125}I-Na were obtained from Amersham International Plc. (Bucks, U.K.). The anti-cyclic AMP serum No. CV-27 pool was supplied by the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and U.S. Department of Agriculture, U.S.A. The doses of drugs were expressed in their final molar concentrations in the flask.

Statistical analysis

All values are given as the mean ± s.e.mean. In some cases, the means of treatment were tested for homogeneity by a two-way analysis of variance, and the difference between specific means was tested for significance by Duncan's multiple-range test (Steel & Torrie, 1960). In other cases, Student's *t*-test was employed. A difference between two means was considered statistically significant when $P<0.05$.

Results

Effects of digoxin on the plasma testosterone concentration

Injection of hCG produced a significant rise in plasma testosterone concentration and the values at 30 and 60 min after the injection (1.04 ± 0.12–2.01 ± 0.13 ng ml^{-1}, $n=8$) were significantly ($P<0.05$ or $P<0.01$) greater than those at $t=0$ min (0.48 ± 0.07 ng ml^{-1}, $n=8$) (Figure 1). Plasma testosterone was unchanged 30 min after intravenous injection of digoxin (1 μg kg^{-1}). However, 60 to 180 min following digoxin injection, the mean concentration of plasma testosterone was reduced by 60% (0.18 ± 0.02 ng ml^{-1} at 180 min, $n=8$, versus 0.45 ± 0.07 ng ml^{-1} at 0 min, $n=8$, $P<0.01$). In the 0.5 to 24 h following coinjection of digoxin and hCG plasma testosterone concentrations (0.76 ± 0.09–1.62 ± 0.29 ng ml^{-1}, $n=8$) were significantly ($P<0.05$ or $P<0.01$) greater than those at $t=0$ min (0.44 ± 0.07 ng ml^{-1}, $n=8$). However, the maximal plasma testosterone levels attained were significantly lower at 120 min (1.60 ± 0.05 ng ml^{-1}, $n=8$, $P<0.05$), and 1440 min (0.76 ± 0.09 ng ml^{-1}, $n=7$, $P<0.05$) than those attained with hCG alone (2.09 ± 0.13 ng ml^{-1} at 120 min, and 1.04 ± 0.12 ng ml^{-1} at 1440 min, $n=8$).

Effects of digoxin on testosterone and cyclic AMP production in vitro

As compared with control group, digoxin (10^{-7}–10^{-4} M) caused a concentration-dependent inhibition of testosterone release from testicular interstitial cells (0.10 ± 0.04–1.86 ± 0.26 ng mg^{-1} protein h^{-1}, $n=8$, versus control group 2.77 ± 0.25 ng mg^{-1} protein h^{-1}, $n=8$, $P<0.05$ or $P<0.01$) (Figure 2). Incubation of testicular interstitial cells with hCG (0.05 iu ml^{-1}) for 1 h produced a significant increase in testosterone secretion (hCG-treated group 57.23 ± 9.68 ng mg^{-1} protein h^{-1}, $n=8$, versus control group 2.77 ± 0.25 ng mg^{-1} protein h^{-1}, $n=8$, $P<0.01$). The responses to hCG were reduced significantly when digoxin (10^{-6}–10^{-4} M) was included in the medium (1.38 ± 0.43–30.95 ± 4.94 ng mg^{-1} protein h^{-1}, $n=8$, versus hCG-treated

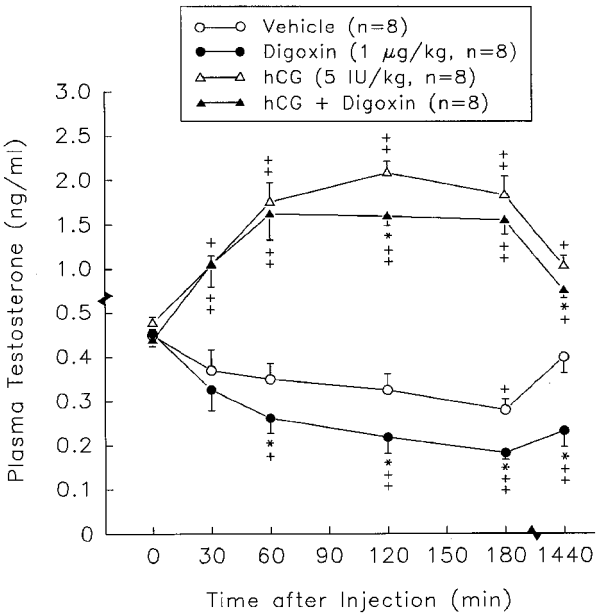

Figure 1 Effects of digoxin on the basal and hCG-stimulated concentration of plasma testosterone in male rats. Rats were given a single intravenous injection of vehicle, digoxin (1 μg ml^{-1} kg^{-1}), hCG (5 iu ml^{-1} kg^{-1}), or hCG plus digoxin *via* right jugular vein. Blood samples were collected *via* the jugular catheter at time indicated after injection. Each value represents mean ± s.e.mean. $^{*}P<0.05$ 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.

group $0.10\pm0.04-0.78\pm0.27$ ng mg^{-1} protein h^{-1}, $n=8$, or $P<0.01$).

In the presence of IBMX, administration of hCG produced a significant approximately 3 fold increase in cyclic AMP accumulation in testicular interstitial cells (30.22 ± 1.11 fmol mg^{-1} protein h^{-1}, $n=8$, versus control group 11.33 ± 0.76 fmol mg^{-1} protein h^{-1}, $n=8$, $P<0.01$) (Figure 3). Digoxin ($10^{-5}-10^{-4}$ M) significantly decreased the content of cyclic AMP in testicular interstitial cells and reduced the rise in cyclic AMP accumulation induced by hCG (digoxin-treated group $7.95\pm0.61-8.56\pm0.62$ ng mg^{-1} protein h^{-1}, $n=8$, versus control group 11.33 ± 0.76 ng mg^{-1} protein h^{-1}, $n=8$, $P<0.01$; digoxin + hCG treated group $11.79\pm0.57-16.98\pm3.02$ ng mg^{-1} protein h^{-1}, $n=8$, versus hCG group 30.22 ± 1.11 ng mg^{-1} protein h^{-1}, $n=8$, $P<0.01$). By contrast, neither the secretion of testosterone nor the production of cyclic AMP in rat testicular interstitial cells was altered by the administration of ouabain in concentrations of $10^{-7}-10^{-4}$ M (data not shown).

Effects of digoxin on cyclic AMP-related testosterone secretion in vitro

Forskolin (10^{-6} M) and 8-Br-cyclic AMP (10^{-4} M) both resulted in significant increases of testosterone secretion by testicular interstitial cells (forskolin-treated group 166% versus vehicle group 100%, $P<0.05$; 8-Br-cyclic AMP-treated group 1649% versus vehicle group 100%, $P<0.01$) (Figure 4). Digoxin (10^{-4} M) reduced the modest secretory response to forskolin from $165.57\pm26.25\%$ of basal to $31.53\pm7.37\%$ ($P<0.01$). It also reduced markedly the secretory response to 8-Br-cyclic AMP from $1649.46\pm70.45\%$ of basal to $1092.4\pm83.35\%$ ($P<0.01$).

Effects of digoxin on the biosynthesis pathway of testosterone in vitro

In concentrations of 10^{-7} M and 10^{-5} M, the five testosterone precursors tested each increased the production of testosterone by testicular interstitial cells (precursor-treated group $2.50\pm0.34-15.95\pm2.27$ ng mg^{-1} protein h^{-1} at 10^{-7} M, $n=8$, and $23.74\pm2.31-40.06\pm3.71$ ng mg^{-1} protein h^{-1} at 10^{-5} M, $n=8$, versus control group 1.58 ± 0.19 ng mg^{-1} protein h^{-1}, $n=8$, $P<0.05$ or $P<0.01$) (Figure 5). Digoxin at 10^{-4} M decreased the production of testosterone facilitated by 25-OH-C in testicular interstitial cell (0.26 ± 0.14 ng mg^{-1} protein h^{-1}, $n=8$, versus 25-OH-C-treated group (10^{-7} M)

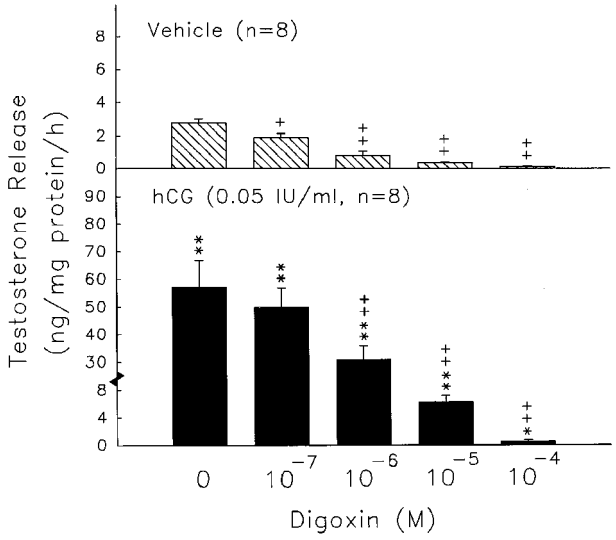

Figure 2 Effects of digoxin ($10^{-7}\sim10^{-4}$ M) on testosterone release *in vitro* from rat testicular interstitial cells pretreated with vehicle or hCG (0.05 iu ml^{-1}). Each column represents mean ± s.e.mean. $^{*}P<0.05$ and $^{**}P<0.01$ compared with vehicle group. $+P<0.05$ and $++P<0.01$ compared with digoxin at 0 M.

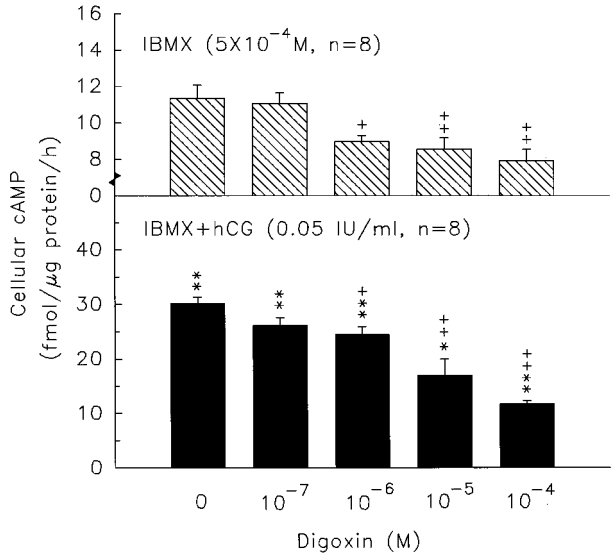

Figure 3 Effects of digoxin ($10^{-7}\sim10^{-4}$ M) on the accumulation of cyclic AMP in rat testicular interstitial cells pretreated with IBMX (5×10^{-4} M), or IBMX + hCG (0.05 iu ml^{-1}). Each column represents mean ± s.e.mean. $^{*}P<0.05$ and $^{**}P<0.01$ compared with IBMX group. $+P<0.05$ and $++P<0.01$ compared with digoxin at 0 M.

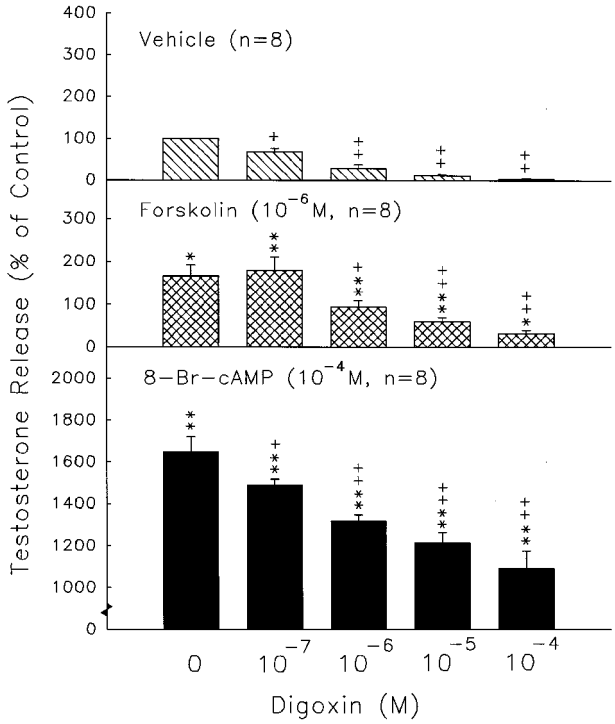

Figure 4 Inhibitory percentile of digoxin ($10^{-7}\sim10^{-4}$ M) on the testosterone release *in vitro* from rat testicular interstitial cells pretreated with vehicle (top), forskolin (10^{-6} M, centre), or 8-Br-cyclic AMP (10^{-4} M, bottom). Each column represents mean ± s.e.mean. $^{*}P<0.05$ and $^{**}P<0.01$ compared with vehicle group. $+P<0.05$ and $++P<0.01$ compared with digoxin at 0 M.

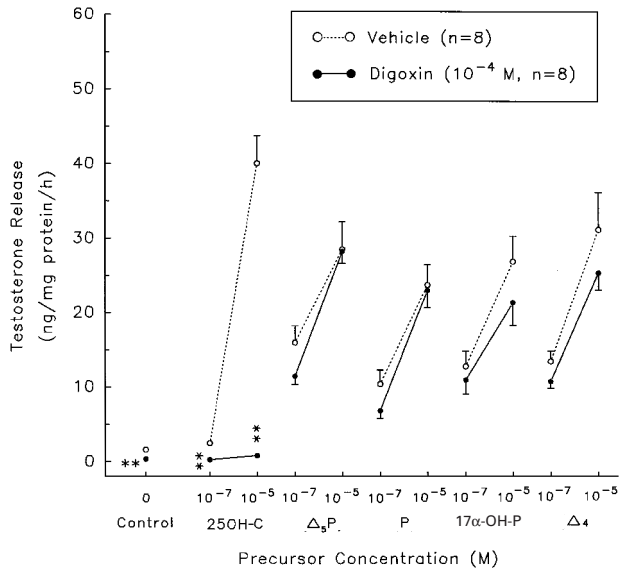

Figure 5 Effects of digoxin (10^{-4} M) on the testosterone release *in vitro* in rat testicular interstitial cells pretreated with vehicle or precursors of steroidogenesis. The precursors included 25-hydroxycholesterol (25-OH-C), pregnenolone (Δ_5P), progesterone (P), 17α-hydroxy-progesterone (17α-OH-P), and androstenedione (Δ_4). Each column represents mean ± s.e.mean. $^{**}P<0.01$ compared with vehicle group.

2.50 ± 0.34 ng mg^{-1} protein h^{-1}, $n=8$, $P<0.01$; 0.83 ± 0.40 ng mg^{-1} protein h^{-1}, $n=8$, versus 25-OH-C-treated group (10^{-5} M) 40.06 ± 3.71 ng mg^{-1} protein h^{-1}, $n=8$, $P<0.01$). However, digoxin did not affect the production of testosterone induced by any of the other four precursors tested.

Discussion

Digoxin has complex direct and indirect actions on the cardiovascular system which are of potential value in the treatment of heart failure and atrial dysrhythmias. Its actions on the reproductive system are less well defined. An early study showed that the plasma testosterone concentrations in healthy men were not altered by the administration of digoxin for 35 days (Kley *et al*., 1982). By contrast, others reported decreased plasma testosterone concentrations in male patients who received digoxin therapy for 2 years (Neri *et al*., 1987). The present data show clearly that *in vitro* digoxin causes a marked, concentration-dependent inhibition of the spontaneous and hCG-stimulated secretion of testosterone by rat testicular interstitial cells (Figure 2). Digoxin also depresses resting and hCG-stimulated plasma testosterone levels when given acutely *in vivo* but, in comparison to the responses observed *in vitro*, these effects were relatively modest, possibly because the dose of digoxin used was small. Taken together, these data suggest that digoxin acts directly on the testis to impair androgen secretion. An early study suggested that long-term digoxin therapy may also depress LH secretion by the pituitary gland (Tappler & Katz, 1979), a phenomenon which would be expected to exacerbate the direct inhibitory actions of the drug on testicular testosterone secretion. Similarly, the digoxin-induced blockade of testosterone synthesis *in vivo* may be reinforced by the concomitant fall of atrial natriuretic peptide (ANP), a peptide which stimulates testosterone secretion *in vitro* in a time and concentration-dependent manner (Mukhopadhyay *et al*., 1986; Foresta & Mioni, 1993).

It is well established that hCG stimulates the secretion of testosterone both *in vivo* (Saez & Forest, 1979; Padron *et al*., 1980; Wang *et al*., 1994; Tsai *et al*., 1996a) and *in vitro* (Simpson *et al*., 1987; Nakhla *et al*., 1989; Wang *et al*., 1994; Tsai *et al*., 1996a; 1997) *via* mechanisms involving increased production of cyclic AMP (Avallet *et al*., 1987; Petersson *et al*., 1988; Sakai *et al*., 1989; Wang *et al*., 1994, Tsai *et al*., 1996a). In accord with these data we observed a marked increase in the cyclic AMP content of cells exposed to hCG. Furthermore, both forskolin and 8-Br-cyclic AMP readily elicited testosterone secretion *in vitro*. The stimulatory effects of forskolin and 8-Br-cyclic AMP, like those of hCG, were dose-dependently attenuated by inclusion of digoxin in the medium (Figure 4). Moreover, digoxin also reduced the rise in cyclic AMP formation induced by hCG (Figure 3). These findings suggest that digoxin inhibits the testosterone secretion by (a) reducing adenylyl cyclase activity and (b) inhibiting the pathways distal to the formation of cyclic AMP which direct cholesterol to the mitochondrial cytochrome $P450_{scc}$ enzyme and thereby promote steroidogenesis *via* the actions of protein kinase A and the steroidogenic acute regulatory (Star) protein. Indeed, our data suggest that cytochrome $P450_{scc}$ (the rate-limiting enzyme of the pathway leading to testosterone biosynthesis) may be a key target for digoxin. Certainly, digoxin reduced markedly the activity of this enzyme but failed to influence the activity of the other steroidogenic enzymes tested. Taken together, these data suggest that digoxin influences at least two key events in the cellular processes leading to testosterone synthesis. The mechanisms by which it produces these effects are unclear. However, interestingly, ouabain (10^{-7}–10^{-4} M), a Na^+/K^+-ATPase inhibitor, which shares many of the pharmacological properties of digoxin failed to reduce the secretion of testosterone *in vivo* or *in vitro* (data not shown), suggesting that the inhibitory actions of digoxin on the testis may be unrelated to its ability to block this ion transporter.

In conclusion, the results demonstrate that digoxin acts directly on the testis to decrease testosterone production. Its actions are effected at least in part by inhibition of cyclic AMP production and cytochrome $P450_{scc}$ activity.

This study was supported by the Grant NRICM-86110 from the National Research Institute of Chinese Medicine, the Grant NSC-88-2314-B-182-072, from the National Science Council, and awards from the Medical Research and Advancement Foundation in memory of Dr Chi-Shuen Tsou, ROC, to P. S. W. Anti-adenosine 3′:5′-cyclic monophosphate antiserum CV-27 pool was kindly supplied by the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and U. S. Department of Agriculture, U.S.A.

References

AVALLET, O., VIGIER, M., PERRARD-SAPOR, M.H. & SAEZ, J.M. (1987). Transforming growth factor β inhibits Leydig cell functions. *Biochem. Biophys. Res. Commun.*, **146,** 574–581.

DIRAMI, G., POULTER, L.W. & COOKE, B.A. (1991). Separation and characterization of Leydig cells and macrophages from rat testes. *J. Endocrinol.*, **130,** 357–365.

FORESTA, C. & MIONI, R. (1993). The role of calcium ions in rat Leydig cells steroidogenesis induced by atrial natriuretic peptide. *Acta. Endocrinol.,* **128,** 274–280.

KLEY, H.K., MULLER, A., PEERENBOOM, H. & KRUSKEMPER, H.L. (1982). Digoxin does not alter plasma steroid levels in healthy men. *Clin. Pharmacol. Ther.,* **32,** 12–17.

KRUMMEN, L.A., WOODRUFF, T.K., COVELLO, R., TAYLOR, R., WORKING, P. & MATHER, J.P. (1994). Localization of inhibin and activin binding sites in the testis during development by *in situ* ligand binding. *Biol. Reprod.,* **50,** 734–744.

LOWRY, O.H., ROSENBOROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

LU, S.S. LAU, C.C., TUNG, Y.F., HUANG, S.W., CHEN, Y.H., SHIH, H.C., TSAI, S.C., LU, C.C., WANG, S.W., CHEN, J.J., CHIEN, E.J., CHIEN, C.H. & WANG, P.S. (1996). Lactate stimulates progesterone secretion via an increase in cAMP production in excised female rats. *Am. J. Physiol.,* **271,** E910–E915.

MUKHOPADHYAY, A.K., BOHNET, H.G. & LEIDENBERGER, F.A. (1986). Testosterone production by mouse Leydig cells is stimulated *in vitro* by atrial natriuretic factor. *FEBS,* **202,** 111–116.

NAKHLA, A.M., BARDIN, C.W., SALOMON, Y., MATHER, J.P. & JANNE, O.A. (1989). The actions of calcitonin on the TM3 Leydig cell line and on rat Leydig cell-enriched cultures. *J. Androl.,* **10,** 311–320.

NERI, A., ZUKERMAN, Z., AYGEN, M., LIDOR, Y. & KAUFMAN, H. (1987). The effect of long-term administration of digoxin on plasma androgens and sexual dysfunction. *J. Sex. Marital. Ther.,* **13,** 58–63.

PADRON, R.S., WISCHUSEN, J., HUDSON, B., BURGER, H.G. & DE KRETSER, D.M. (1980). Prolonged biphasic response of plasma testosterone to single intramuscular injections of human chorionic gonadotropin. *J. Clin. Endocrinol. Metab.,* **50,** 1100–1104.

PAYNE, A.H. & O'SHAUGHNESSY, P.J. (1996). Structure, function and regulation of steroidogenic enzymes in the Leydig cell. In: *The Leydig Cell.* ed. Payne, A.H., Hardy, M.P. & Russell, L.D. chap, 12, pp. 259–286. Vienna: Cache River Press.

PAYNE, A.H. & SHA, L. (1991). Multiple mechanisms for regulation of 3β-hydrosteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$-isomerase, 17$\alpha$-hydroxylase/C17-20 lyase cytochrome P450 messenger ribonucleic acid levels in primary cultures of mouse Leydig cells. *Endocrinology,* **129,** 1429–1453.

PETERSSON, F., ANDERSSON, R.G.G., BERG, A.A.S. & HAMMAR, M. (1988). Early effects of hCG on human testicular cyclic AMP content, protein kinase activity, *in vitro* progesterone conversion and the serum concentrations of testosterone and oestradiol. *Int. J. Androl.,* **11,** 179–186.

SAEZ, J.M. & FOREST, M.G. (1979). Kinetics of human chorionic gonadotropin-induced steroidogenic response of the human testis. I. Plasma testosterone: Implications for human chorionic gonadotropin stimulation test. *J. Clin. Endocrinol. Metab.,* **49,** 278–283.

SAKAI, A., SAKAKIBARA, R. & ISHIGURO, M. (1989). Human chorionic gonadotropin-rich A chain hybrid protein: A hormone analog for the study of signal transduction. *J. Biochem.,* **105,** 275–280.

SHEU, W.J., PU, H.F., WANG, S.W., HO, L.L.T. & WANG, P.S. (1987). Metabolic clearance rate and secretion rate of gastric inhibitory polypeptide in the rat. *Chinese J. Physiol.,* **30,** 25–33.

SIMPSON, B.J.B., WU, F.C.W. & SHAPRE, R.M. (1987). Isolation of human Leydig cells which are highly responsive to human chronic gonadotropin. *J. Clin. Endocrinol. Metab.,* **65,** 415–422.

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

STOFFER, S.S., HYNES, K.M., JIANG, N.S. & RYAN, R.J. (1973). Digoxin and abnormal serum hormone levels. *JAMA.,* **225,** 1643–1644.

TAPPLER, B. & KATZ, M. (1979). Pituitary-gonadal dysfunction in low-output cardiac failure. *Clin. Endocrinol.,* **10,** 219–226.

THOREUX-MANLEY, A., LE GOASCOGNE, C., SEGRETAIN, D., JÉGOU, B. & PINON-LATAILLADE, G. (1995). Lead affects steroidogenesis in rat Leydig cells *in vivo* and *in vitro*. *Toxicology,* **103,** 53–62.

TSAI, S.C., CHEN, J.J., CHIAO, Y.C., LU, C.C., LIN, H., YEH, J.Y., LO, M.J., KAU, M.M., WANG, S.W. & WANG, P.S. (1997). The role of cyclic AMP production, calcium channel activation and enzyme activities in the inhibition of testosterone secretion by amphetamine. *Brit. J. Pharmacol.,* **122,** 949–955.

TSAI, S.C., CHIAO, Y.C., LU, C.C., DOONG, M.L., CHEN, Y.H., SHIH, H.C., LIAW, C., WANG, S.W. & WANG, P.S. (1996a). Inhibition by amphetamine of testosterone secretion through a mechanism involving an increase of cyclic AMP production in rat testes. *Brit. J. Pharmacol.,* **118,** 984–988.

TSAI, S.C., LU, C.C., LAU, C.P., HWANG, G.S., LEE, H.Y., CHEN, S.L., HUANG, S.W., SHIH, H.C., CHEN, Y.H., CHIAO, Y.C., WANG, S.W. & WANG, P.S. (1996b). Progesterone stimulates *in vitro* release of prolactin and thyrotropin involving cAMP production in rat pituitary. *Chinese J. Physiol.,* **39,** 245–251.

WANG, P.S., LIU, J.Y., HWANG, C.Y., HWANG, C., DAY, C.H., CHANG, C.H., PU, H.F. & PAN, J.T. (1989). Age-related differences in the spontaneous and thyrotropin-releasing hormone stimulated release of prolactin and thyrotropin in ovariectomized rats. *Neuroendocrinology,* **49,** 592–596.

WANG, P.S., TSAI, S.C., HWANG, G.S., WANG, S.W., LU, C.C., CHEN, J.J., LIU, S.R., LEE, K.Y., CHIEN, E.J., CHIEN, C.H., LEE, H.Y., LAU, C.P. & TSAI, C.L. (1994). Calcitonin inhibits testosterone and luteinizing hormone secretion through a mechanism involving an increase in cAMP production in rats. *J. Bone Miner. Res.,* **9,** 1583–1590.

(Received April 6, 1998
Revised September 1, 1998
Accepted September 14, 1998)