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THE EFFECT OF KETOCONAZOLE ON ADRENAL AND TESTICULAR STEROIDOGENESIS *IN VITRO*

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Abstract—(1) The effect of the anti-fungal agent, ketoconazole, on cortisol secretion from adrenal cells stimulated with ACTH (1–24, 50 ng/l) and on testosterone secretion from Leydig cells stimulated with LH (5 i.u./l), has been tested. The concentration of drug which inhibited cortisol and testosterone secretion by 50% (ED_{50}) was 3.6 ± 0.7 μmol/l and 0.61 ± 0.03 μmol/l, respectively. (2) The effect of ketoconazole on adrenal and testicular steroidogenesis was completely reversible. Thus, adrenal and testicular cells which had been washed after exposure to >95% inhibitory dose of ketoconazole responded in a similar manner to hormone stimulation as cells similarly washed and which had not been exposed to drug. (3) The sites of the anti-steroidogenic effect of ketoconazole have been established using a method based upon the sequential stimulation by the exogenous precursor steroids of the various steps leading to the biosynthesis of cortisol by adrenal cells and testosterone by Leydig, cells. We conclude that ketoconazole reversibly inhibits the sequence between ACTH/LH binding and pregnenolone production and also inhibits testicular C-17–C-20, lyase activity.

The imidazole derivative, ketoconazole (Nizoral, Janssen Pharmaceutica, Beerse, Belgium), is an orally-effective broad spectrum anti-fungal agent. This drug inhibits the C-14-demethylation of lanosterol, thus preventing its conversion to ergosterol in fungi and to cholesterol in mammalian cells [1, 2]. Ketoconazole has recently been shown to suppress both adrenal and testicular steroid secretion *in vivo* and *in vitro*. In healthy humans and dogs receiving single doses or long-term therapy, ketoconazole results in a marked but transient reduction in plasma testosterone [3–6]. Although clinically adverse side effects are rare, there have been reports on the development of gynaecomastia in male patients on long-term treatment [7]. The drug has been shown to be a reversible inhibitor of testosterone synthesis by dispersed Leydig cells [4, 8], inhibiting the enzyme C-17–C-20 lyase in the androgen biosynthetic pathway [3, 6, 8, 9]. High dose therapy has been shown to produce long-term suppression of androgen production in patients with prostatic carcinoma [10] and long-term treatment has been shown to impair the fertility of male rats by decreasing sperm number and motility and increasing the number of abnormal sperm [11]. In healthy individuals the cortisol response to ACTH was significantly blunted after administration of a therapeutic concentration of ketoconazole [12] and the drug-induced suppression of corticosteroid secretion has been reported to result from inhibition of the mitochondrial P-450 enzymes, cholesterol desmolase and 11β hydroxylase [13, 14]. Ketoconazole has been used clinically in the treatment of Cushing's syndrome [15].

We have recently developed two *in vitro* systems which have enabled us to estimate the biopotency and site of action of various drugs affecting adrenal steroidogenesis [16–18]. In this study we have employed similar techniques to estimate the relative biopotency of ketoconazole as an inhibitor of adrenal and testicular steroidogenesis. The reversibility of this inhibition has been examined. In addition we have delineated the enzymic sites of action in both the cortisol and androgen biosynthetic pathways.

MATERIALS AND METHODS

Preparation of isolated Leydig cells. The experimental procedure is a modified version [18] of that originally described by Van Damme *et al.* [19]. Briefly, the testes from two mice were removed, decapsulated and cut into small pieces using fine scissors. The cells were dispersed by mechanical agitation for 10 min at 37° and the cell suspension filtered through nylon (100 μ) mesh, washed in Dulbecco's minimum essential medium (DMEM, Flow Laboratories, Irvine, Scotland) and resuspended at 2×10^6 cells/ml. The cell suspension was then preincubated for 1 hr at 37° before a further centrifugation and resuspension.

In all experiments aliquots (100 μl) of cell suspension were dispensed into a 48-well tissue culture plate. The total volume of incubate in each well was 200 μl and the final cell concentration was 0.33×10^6 cells/ml. Drugs and steroids were incorporated into the cell suspension in dimethylsulphoxide (DMSO) such that the final concentration of solvent was 2.5%. DMSO (2.5%) was also included in the control (no added LH) and LH (5 i.u./l)-stimulated cells in the absence of drug or exogenous steroid.

In the first set of experiments the cells were stimulated for 2 hr at 37° with luteinizing hormone (LH, 5 i.u./l, 1st IRP code 68/40) either alone or in com-

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bination with increasing concentrations of ketoconazole (5×10^{-8}–2.5×10^{-5} mol/l). At the end of the incubation, duplicate samples were taken and assayed for testosterone by radioimmunoassay. Ketoconazole did not interfere in the assay at the concentration employed.

In another set of experiments the cells were stimulated for 2 hr at 37° with LH (5 i.u./l) either alone or in combination with ketoconazole (5×10^{-6} mol/l). At the end of the incubation, duplicate samples were taken for estimation of testosterone. The cells which had and had not been exposed to ketoconazole were then washed twice in DMEM and re-stimulated with LH 5 i.u./l for a further 2 hr at 37°. At the end of this incubation duplicate samples were again taken for measurement of testosterone.

To determine the site of action of ketoconazole in the androgen biosynthetic pathway, the cells were challenged with the testosterone precursor steroids (all at 7.5×10^{-6} mol/l) pregnenolone (Pe), 17-hydroxypregnenolone (17-Pe), progesterone (Po), 17-hydroxyprogesterone (17-Po), dehydroepiandrosterone (DHEA), androstenedione (Ao), androstenediol (A-diol), or with either dibutyryl cAMP (10^{-3} mol/l) or LH (5 i.u./l) in the absence or presence of ketoconazole (1.25×10^{-5} mol/l). After 2 hr incubation at 37°, duplicate samples were taken for estimation of testosterone.

Preparation of isolated guinea-pig adrenal cells. Dispersed guinea-pig adrenal cells were prepared as described previously [20, 21] except that collagenase was used instead of trypsin. Briefly, the adrenals from a male guinea-pig were chopped into 1-mm cubes using a McIlwain tissue chopper, the tissue pieces washed with Eagle's minimum essential medium (EMEM) and dispersed by mechanical agitation in EMEM containing collagenase (2 mg/ml). The cells were collected by centrifugation (300 *g* for 5 min) and washed twice with Eagle's medium containing bovine serum albumin (BSA 0.5%), calcium (8×10^{-3} mol/l) and ascorbate (2×10^{-3} mol/l). The above mixture served as incubation medium. Finally, the cell suspension was filtered through nylon (100 μ) mesh. The cells (1×10^6 cells/ml) were then pre-incubated for 2 hr at 37° in an atmosphere of 100% O_2. After pre-incubation the cell suspension was centrifuged to remove any secreted cortisol and resuspended in fresh incubation medium.

In all experiments aliquots (40 μl) of cell suspension were dispensed into a 96-well tissue culture plate. The total volume of incubate in each well was 100 μl and the final cell concentration was 0.75×10^6 cells/ml. Drugs and steroids were incorporated into the cell suspension in DMSO such that the final concentration of solvent was 2.5%. DMSO (2.5%) was also included in the control (no added ACTH) and ACTH-stimulated wells in the absence of drug or exogenous steroid.

In the first set of experiments aliquots (40 μl) of cell suspension were dispensed into a 96-well tissue culture plate and stimulated for 90 min at 37° with 50 ng/l ACTH either alone or in combination with increasing concentrations of ketoconazole. After 90 min incubation duplicate 10-μl samples were taken and assayed for cortisol by radioimmunoassay.

In a set of experiments designed to examine the reversibility of the inhibition, the cells were stimulated for 90 min at 37° with ACTH (50 ng/l) either alone or in combination with ketoconazole (2.5×10^{-5} mol/l). At the end of the incubation duplicate samples were taken for estimation of cortisol. The cells which had and had not been exposed to drug were then washed twice in EMEM and re-stimulated with ACTH 50 ng/l for a further 90 min at 37°. At the end of this incubation duplicate samples were again taken for measurement of cortisol.

To determine the site of action of ketoconazole in the cortisol biosynthetic pathway, the cells were challenged with the cortisol precursor steroids (all at 10^{-5} mol/l) Pe, 17-Pe, Po, 17-Po and 11-deoxycortisol, or with either dibutyryl cAMP (10^{-3} mol/l) or ACTH (100 ng/l) in the absence or presence of ketoconazole (1.25×10^{-5} mol/l). After 90 min incubation at 37°, duplicate samples were taken for estimation of cortisol.

Source of chemicals. LH (1st IRP code 68/40, 77 i.u. per ampoule) was supplied by National Institute of Biological Standards and Control, Hampstead, London. ACTH (1–24) was kindly donated by Dr C. McMartin, Ciba-Geigy, Horsham, West Sussex, U.K. Ketoconazole was supplied by Janssen Pharmaceuticals, Marlow, Bucks, U.K. Dimethylsulphoxide (AR) was purchased from Fisons plc, Loughborough, U.K.

RESULTS

Estimation of the biopotency of ketoconazole

The effect of increasing concentrations of ketoconazole on LH (5 i.u./l)-stimulated testosterone secretion and ACTH (50 ng/l)-stimulated cortisol secretion is shown in Fig. 1. Inhibition of testosterone secretion occurred over a much lower concentration range (2.5×10^{-7}–2.5×10^{-6} mol/l ketoconazole) than inhibition of cortisol secretion (1×10^{-6}–2.5×10^{-5} mol/l). The concentration of drug which inhibited cortisol and testosterone secretion by 50% (ED_{50}) was (mean ± SE, N = 3) 3.6 ± 0.7 μmol/l and 0.61 ± 0.03 μmol/l, respectively.

Reversibility of inhibition by ketoconazole

ACTH (50 ng/l)-stimulated cortisol secretion and LH (5 i.u./l)-stimulated testosterone secretion from adrenal and Leydig cells which had been washed twice after exposure to >95% inhibitory dose of ketoconazole are shownin Figs 2(a) and (b). ACTH-stimulated cortisol production from the washed control cells was only 43% of that from freshly incubated cells. In contrast LH-stimulated testosterone secretion from washed control cells was not significantly different from that of freshly incubated cells. Both adrenal and testicular cells which had been washed after exposure to ketoconazole responded in a similar manner to hormone stimulation as washed cells which had not been exposed to drug.

Site of action of ketoconazole

Figure 3(a) shows ACTH-stimulated, basal, dibutyryl cAMP and steroid precursor (Pe, 17-Pe, Po, 17-Po and 11-deoxycortisol) provoked cortisol

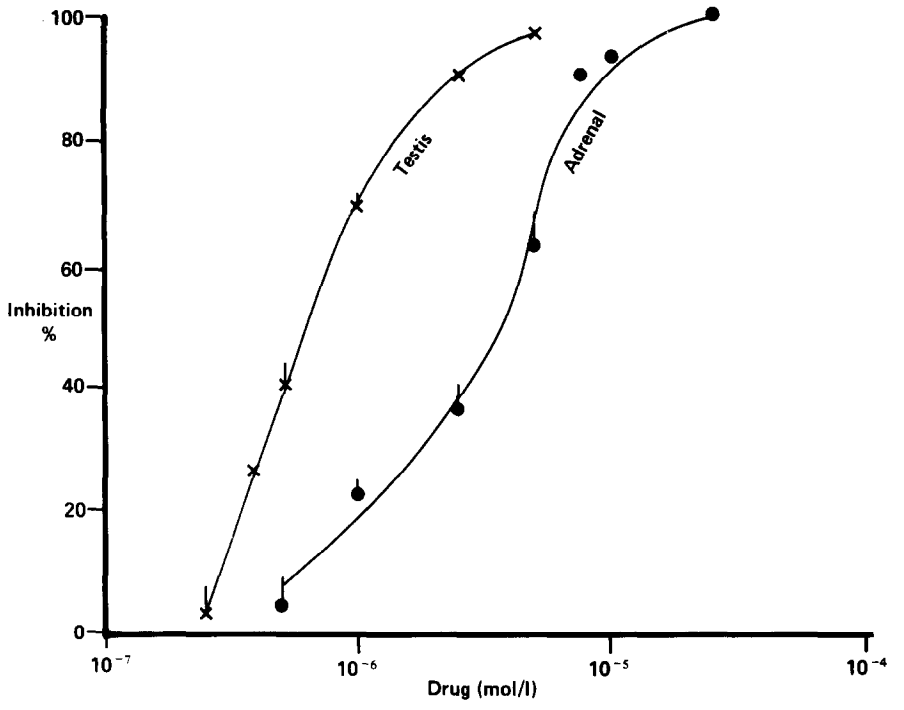

Fig. 1. Inhibition of ACTH (50 ng/l)-stimulated cortisol secretion by guinea-pig adrenal cells (●) and LH (5 i.u./l) stimulated testosterone secretion by mouse Leydig cells (×) by ketoconazole. Each point is the mean ± SEM from three separate experiments.

secretion by adrenal cells in the absence and presence of ketoconazole (1.25×10^{-5} mol/l). Steroid precursors, dibutyryl cAMP or ACTH increased cortisol secretion >7-fold over that secreted by adrenal cells in their absence. ACTH and dibutyryl cAMP-stimulated cortisol secretion was inhibited by >90% by ketoconazole. In contrast, the steroid-provoked cortisol secretion was unaffected by drug.

Leydig cells were similarly challenged with: (a) the testosterone precursor steroids (Pe, 17-Pe, Po 17-Po, dehydroepiandrosterone (DHEA), androstenedione (Ao) and androstenediol (A-diol); (b) dibutyryl cAMP (10^{-3} mol/l); or (c) LH (5 i.u./l) in the absence and presence of ketoconazole (1.25×10^{-5} mol/l) [Fig. 3(b)]. Steroid precursors, dibutyryl cAMP or LH, increased testosterone secretion >18-fold over that secreted by Leydig cells incubated in their absence. LH- and dibutyryl cAMP-stimulated testosterone secretion was inhibited by >84% by ketoconazole. Pe- and 17-Pe-stimulated testosterone secretion was inhibited by >66%, and Po- and 17-Po-stimulated secretion was inhibited >45%. There was no inhibition of DHEA-, Ao- and A-diol-provoked testosterone secretion.

DISCUSSION

In this study we have demonstrated that ketoconazole is able to depress ACTH-stimulated cortisol secretion from adrenal cells and LH-stimulated testosterone secretion from testicular cells in a dose-dependent manner. Ketoconazole is more potent as an inhibitor *in vitro* of testicular (ED_{50} 0.61 μmol/l) rather than adrenal (ED_{50} 3.6 μmol/l) steroidogenesis, but inhibition is completely reversible in both cell types. After a single oral dose of 200 mg, ketoconazole reaches peak plasma levels of 3–4.5 μg/ml, i.e. 5.6–8.5 μmol/l [22] which exceed the *in vitro* ED_{50} values for inhibition of both adrenal and testicular steroidogenesis. In general, the relevance of the anti-steroidogenic potency of a drug measured in any *in vitro* system must be viewed with some caution, but in the absence of clinical or other evidence from animal studies, it may give some indication as to possible *in vivo* activity. In the case of ketoconazole, evidence for suppression of both adrenal and testicular steroid secretion *in vivo* has been reported [5, 6, 12, 15].

The ED_{50} of 0.61 μmol/l for ketoconazole induced inhibition of testosterone secretion is in good agreement with the value of 0.55 μmol/l reported by De Coster [8] who also found inhibition of testosterone synthesis to be totally reversible. The ED_{50} of 3.6 μmol/l for ketoconazole-induced inhibition of cortisol secretion is similar to the value of 3.5 μmol/l reported using this method for the established anti-adrenal drug, metyrapone [16]. However, ketoconazole was found to be a less potent inhibitor of adrenal corticosteroidogenesis in the present study than in two studies using rat adrenal cells and mouse adrenal cortex tumour cells where an ED_{50} value of 0.56 μmol/l was reported in both cases [12, 23]. In agreement with the present results, both studies found the inhibitory effects of ketoconazole to be rapidly reversible.

The sites of the anti-steroidogenic effect of keto-

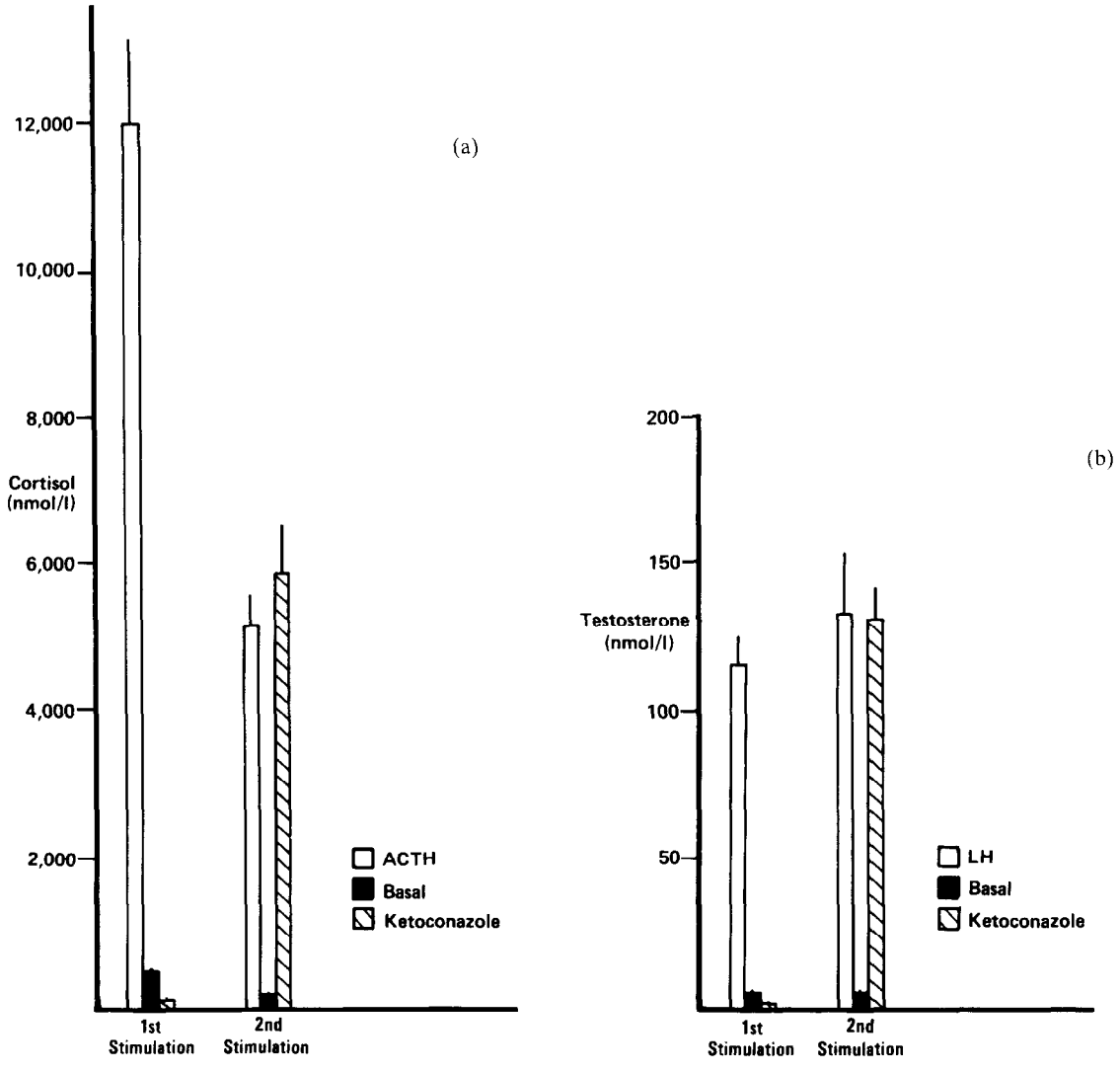

Fig. 2. (a) Cortisol secretion from guinea-pig adrenal cells stimulated for 90 min at 37° with ACTH (50 ng/l, open column), no added ACTH (basal, solid column), and ketoconazole (2.5×10^{-5} mol/l, hatched column) is shown (first stimulation). The cells which had and had not been exposed to drug were then washed twice in EMEM and restimulated with ACTH (50 ng/l) for a further 90 min at 37° (second stimulation). (b) Testosterone secretion from mouse Leydig cells stimulated for 2 hr at 37° with LH (5 i.u./l, open column), no added LH (basal, solid column) and ketoconazole (5×10^{-6} mol/l, hatched column) is shown (first stimulation). The cells which had and had not been exposed to ketoconazole were then washed twice in DMEM and re-stimulated with LH (5 i.u./l) for a further 2 hr at 37° (second stimulation).

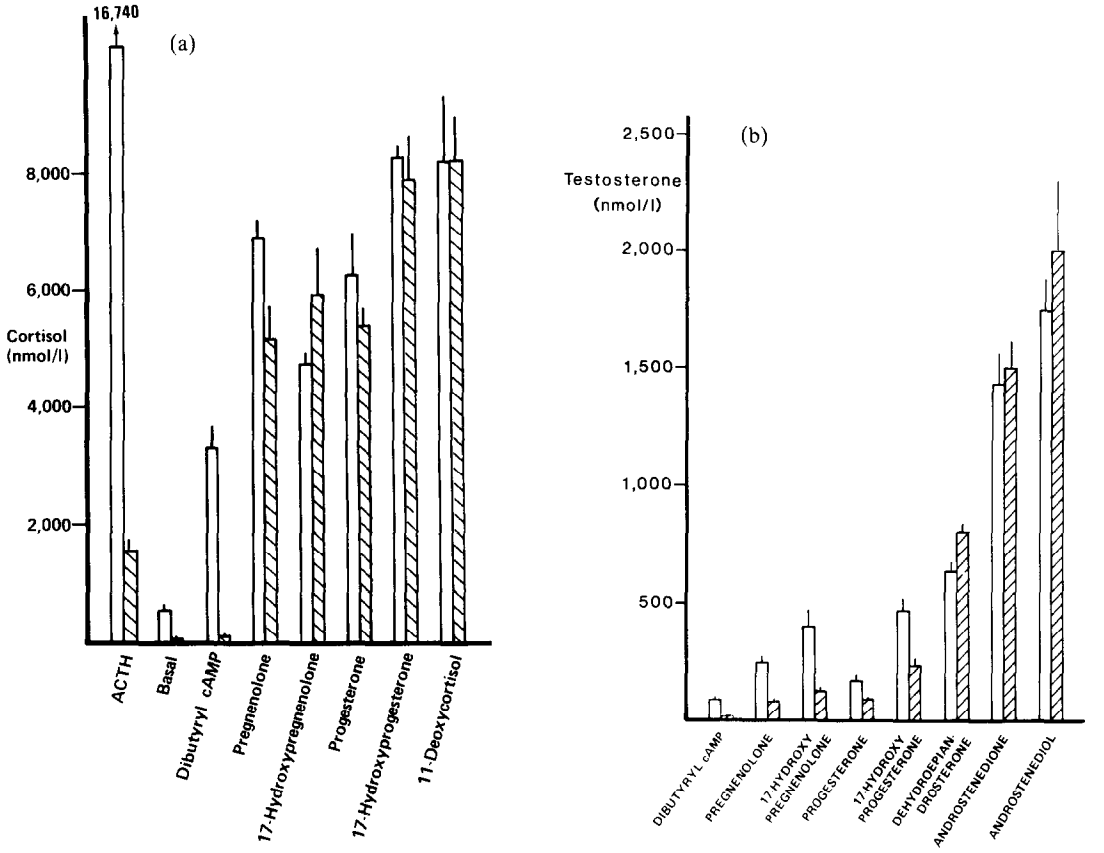

Fig. 3. (a) Cortisol secretion from guinea-pig adrenal cells is shown in response to ACTH (1–24, 100 ng/l), no added ACTH (basal), dibutyryl cAMP (10^{-3} mol/l) or the cortisol precursor steroids (all at 10^{-5} mol/l) pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone or 11-deoxycortisol in the presence (hatched columns) and absence (open columns) of ketoconazole (1.25×10^{-5} mol/l). (b) Testosterone secretion from mouse Leydig cells is shown in response to dibutyryl cAMP 10^{-3} mol/l) or the testosterone precursor steroids (all at 7.5×10^{-6} mol/l) pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, androstenediol in the presence (hatched columns) and absence (open columns) of ketoconazole (1.25×10^{-5} mol/l). Columns represent the mean ± SD (N = 4).

conazole have been delineated using a method based upon the sequential stimulation of the various steps leading to the biosynthesis of cortisol by adrenal cells and testosterone by Leydig cells. We conclude that on the basis of the known biosynthetic pathway for cortisol (Fig. 4) and testosterone (Fig. 5) that ketoconazole inhibits the sequence between ACTH/LH binding and pregnenolone production and also inhibits testicular C-17–C-20 lyase activity. The inhibition of adrenal steroidogenesis between ACTH binding and pregnenolone production confirms the findings of previous studies [13, 14, 23]. However, in contrast to the present results an additional site of action at the cytochrome P-450 dependent enzyme, 11β-hydroxylase, has been demonstrated *in vitro* by Loose *et al.* [13] and *in vivo* by Engelhardt *et al.* [14]. In the study by Loose *et al.* isolated rat adrenal cells were pretreated with ketoconazole for 15 min before being challenged with radiolabelled cortisol precursor substrates. Analysis of products was performed by high performance liquid chromatography. Since similar concentrations of drug were employed in the present study (1.25×10^{-5}mol/l) and in the study by Loose *et al.* (0.94×10^{-5} mol/l), the reasons for this discrepancy remain unclear.

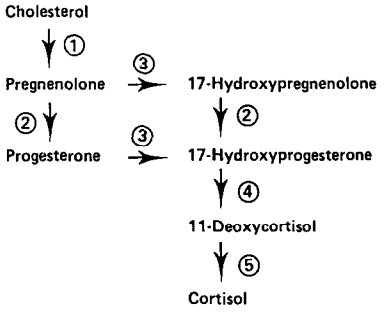

Fig. 4. The biosynthesis of cortisol. (1) Cholesterol desmolase; (2) Δ^5, 3β-hydroxysteroid dehydrogenase; (3) 17α-hydroxylase; (4) 21-hydroxylase; (5) 11β-hydroxylase.

The data presented in this paper confirm the observation that *in vitro* ketoconazole inhibits testosterone biosynthesis by acting at C-17–C-20 lyase [3, 6, 8, 9]. At high concentrations of ketoconazole (10–300 μg/

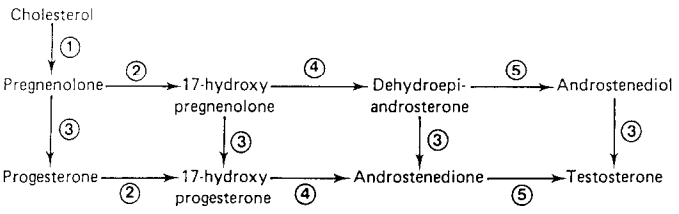

Fig. 5. The biosynthesis of testosterone. (1) Cholesterol desmolase; (2) 17 α-hydroxylase (3) Δ^5, 3β-hydroxysteroid dehydrogenase; (4) C-17–C-20 lyase; (5) 17β-hydroxysteroid dehydrogenase.

ml, i.e. 2.4×10^{-5}–7.2×10^{-4} mol/l) an additional site of action at 17α-hydroxylase has also been reported [9], although at the concentration used in this study (i.e. 1.25×10^{-5} mol/l) no inhibition of this enzyme was apparent.

In conclusion, ketoconazole is approximately six times more potent an inhibitor of testicular than adrenal steroidogenesis and in both cases drug-induced inhibition is readily reversible. This anti-fungal agent exerts its inhibitory effects at the sequence between ACTH/LH binding and pregnenolone production and also inhibits testicular C-17–C-20 lyase activity.

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