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KETOCONAZOLE INHIBITION OF THE BIFUNCTIONAL CYTOCHROME P450c17 DOES NOT AFFECT ANDROGEN FORMATION FROM THE ENDOGENOUS LYASE SUBSTRATE

THE CATALYTIC SITE REMAINS REFRACTORY IN THE COURSE OF INTERMEDIARY HYDROXYPROGESTERONE PROCESSING

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Abstract—The inhibition of the bifunctional steroidogenic cytochrome P450c17 (CYP17: steroid-17α-hydroxylase/steroid-17,20-lyase) by the imidazole-type fungicide, {(±)-*cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine} (ketoconazole), was investigated with the aim of differentiating between effects on androgen formation from exogenously added and endogenously produced 17α-hydroxyprogesterone. Using microsomal membranes from rat testis, turnover of progesterone by P450c17 was competitively inhibited by ketoconazole with $K_I = 0.40\ \mu M$. Ketoconazole did not affect the linear relationship between the ratio of productive events (corresponding to androgen formation rates) versus abortive events (corresponding to 17α-hydroxyprogesterone formation rates) and the sum of catalytic events. This was an indication that this inhibitor did not interfere with intermediate processing by P450c17. Androgen formation from exogenous but not from endogenous 17α-hydroxyprogesterone was competitively inhibited by ketoconazole. The simultaneous conversion of 1 μM each of [^{3}H]progesterone and 17α-hydroxy[^{14}C]progesterone was also reduced by ketoconazole. Calculation of $^3H/^{14}C$ ratios in the 17α-hydroxyprogesterone and androgen fractions revealed that the endogenous 17α-hydroxyprogesterone pool was metabolized to androgens at rates 6.4, 11.6, 17.6 and 21.2-fold faster than the exogenous pool in the presence of 0.5, 1, 2 and 4 μM ketoconazole, respectively; this value was only 4.0 in controls. It is concluded that ketoconazole inhibits turnover of steroid ligands only when they approach the P450c17 active site in a substrate-state and that inhibition of androgen formation from progesterone is due to inhibition of the first catalytic step only. A model is described in which the P450c17 active site is refractory towards ketoconazole when the intermediary steroid is retained and being processed at that site.

There is considerable interest in the inhibition of steroid hormone-synthesizing cytochromes P450 (CYPs) since this may represent an approach to the treatment of steroid-sensitive (e.g. prostate and breast cancer) and steroid-producing (e.g. adrenal carcinoma) tumours [1–4]. Ketoconazole {(±)-*cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]-phenyl]piperazine} has been intensively studied in this connection. It was initially introduced as an orally active, broad spectrum, imidazole-type, antifungal agent which acts through inhibition of P450-14DM (CYP51) catalysing the lanosterol 14α-methyl demethylase reaction cascade [3–6]. This results in a depression of yeast and fungal ergosterol biosynthesis, whereas mammalian cholesterol biosynthesis is much less sensitive towards ketoconazole inhibition. At higher doses, ketoconazole was found to inhibit also a variety of mammalian cytochromes P450 either involved in steroid hormone biosynthesis, such as P450scc (CYP11A: cholesterol monooxygenase or side-chain cleavage system or pregnenolone synthetase) [7, 8], P450c11 (CYP11B: steroid-11β-hydroxylase) [8, 9], P450c17 (CYP17: steroid-17α-hydroxylase/steroid-17,20-lyase) [7, 8, 10–13], P450arom (CYP19: aromatase or estrogen synthetase) [12, 14] and P450c21 (CYP21A: steroid-21-hydroxylase) [7, 11, 12], or involved in hepatic steroid hormone metabolism [15, 16]. Though acting non-specifically, ketoconazole inhibits individual cytochrome P450 enzymes to different extents; the IC_{50} or K_I values decrease in the order CYP19 = CYP21A ≫ CYP11A > CYP11B > CYP17 [4, 17, 18]. On the basis of these results, ketoconazole has been considered as a possible therapeutic agent for lowering pathologically elevated steroid hormone levels [3], and it has been used successfully in the treatment of Cushing's syndrome [19] and prostatic carcinoma [20]. The discovery of differential inhibition profiles of azole derivatives towards specific P450 enzymes has initiated a search for even more specific inhibitors of steroid-synthesizing cytochromes P450 [3, 4].

Several of the cytochromes P450 involved in

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catalysis of steroid-synthesizing metabolic pathways, especially the isoforms CYP11A [21], CYP17 [22], CYP19 [23] and CYP51 [6], have each been claimed to possess two or three catalytic functions [1]. Amongst these, the bifunctional P450c17 (EC 1.14.99.9/EC 4.1.2.30) is of special importance since it is located at a key branch-point in steroidogenic pathways: in the rat, its hydroxylase activity yields 17α-hydroxyprogesterone which may be further processed in the adrenals to glucocorticoids, whereas the synchronized action of both its hydroxylase and lyase activities yields androstenedione (preferentially in the testes) which may be converted further to either testosterone or estrone [22, 24–26]. Thus, *specific* inhibition of testosterone production, which should be beneficial, for instance, for patients suffering from androgen-dependent prostate carcinoma, requires either a compound which inhibits the lyase but not the hydroxylase function of P450c17 [22, 24], or a compound which inhibits the testicular but not the adrenal enzyme [25, 26]. Though it has been demonstrated unequivocally that both activities are catalysed by one single polypeptide chain containing one heme [26, 27], the observation that the hydroxylase/lyase ratio is not necessarily constant [22, 25, 26] opens the possibility that both P450c17 activities may under certain conditions be influenced differently.

Since exogenously added and endogenously (via the hydroxylase activity of P450c17) formed 17α-hydroxyprogesterone is not metabolized equally by the P450c17-lyase activity, at least in testicular tissue [28, 29], an enzyme kinetic analysis of both the hydroxylase/lyase sequence on progesterone as the substrate and the lyase reaction on 17α-hydroxyprogesterone as the substrate, combined with the follow-up of simultaneous conversion of both steroids in a dual-label/dual-substrate experiment, was used to differentiate between the two P450c17 functions. Using ketoconazole as the model drug to inhibit cytochrome P450c17, the differential sensitivity of the two P450c17 activities to imidazole inhibition was studied. The direct intra-enzyme transfer of endogenous 17α-hydroxyprogesterone to androgen was seen to be much less inhibited by ketoconazole than 17α-hydroxyprogesterone formation from progesterone or androgen formation from exogenous 17α-hydroxyprogesterone. Hence, the primary target of ketoconazole action on P450c17 is proposed to be the enzyme's substrate access/first oxygen insertion site rather than the intermediate-processing/second oxygen insertion site.

MATERIALS AND METHODS

The methodological design, materials and applied methods for the enzyme kinetic and the double-label/double-substrate experiment were based on the procedures described previously [22, 29]. Microsomal membranes were prepared from testes of Han:Wistar rats aged 10–12 weeks by homogenization in 20 mM Tris + 250 mM sucrose buffer (pH 7.4) and repeated differential centrifugation resulting in the washed 150,000 g × 45 min pellet of the 12,000 g × 10 min supernatant. The incubation assays contained, in a final assay volume of 0.5 mL, microsomal membrane suspensions equivalent to 0.7 mg protein/mL [30] and 47 nM cytochrome P450 [31] in 50 mM phosphate buffer (pH 7.4) containing 130 μM NADPH and 280 μM $MgCl_2$. Progesterone (Calbiochem, Frankfurt/Main, F.R.G.) {including 200 Bq/mL [1,2-^{3}H]progesterone (2.0 TBq/mmol; New England Nuclear, Dreieich, F.R.G.)} and 17α-hydroxyprogesterone (Serva, Heidelberg) {including 200 Bq/mL 17α-hydroxy[4-^{14}C]progesterone (1.8 GBq/mmol; New England Nuclear)} with or without ketoconazole [Nizoral® (R41400, Batch No. E2101), kindly provided by Dr W. Karraß, Janssen GmbH, Neuss, F.R.G.] were added in ethanol-containing (final ethanol concentration 0.7% by vol. in all tubes) buffer.

For characterization of ketoconazole effects on P450c17-catalysed progesterone turnover, eight different progesterone concentrations (from 0.12 to 12 μM) and two different inhibitor concentrations (0.5 and 2 μM in addition to control incubations) were employed. Identical ketoconazole concentrations were used to characterize P450c17-catalysed turnover of 17α-hydroxyprogesterone (eight concentrations ranging from 0.25 to 24 μM). All these incubations were performed under initial rate conditions, i.e. up to 1 min with the lowest and up to 20 min with the highest steroid concentrations. In the dual-substrate approach, tubes contained always 1 μM progesterone plus 1 μM 17α-hydroxyprogesterone in the presence of 0.5, 1, 2, 4 μM or no ketoconazole, and the time-course of steroid metabolism was recorded up to 14 min. Incubations were generally performed at 32°, the physiological scrotal testis temperature in the rat. After the times specified, aliquots (200 μL) were transferred to extraction tubes containing 5 mL diethylether. After extraction from the aqueous phase, steroids were separated by TLC as described [32] and eluted from the silica gel with a dichloromethane–methanol (4 + 1 by vol.) mixture. Radioactivities for the ^{3}H and ^{14}C labels in the progesterone, 17α-hydroxyprogesterone and androgen fractions were analysed in an LKB-Wallac 1214 Rackbeta liquid scintillation counter with a dual-label program; androstenedione and testosterone were combined to the androgen fraction since they are in an NADPH/$NADP^+$-controlled equilibrium [22, 29].

In the enzyme kinetic experiments, relative steroid concentrations were converted to steroid formation rates, and the parameters V, K_M and K_I were calculated from transformations based on Eadie–Hofstee diagrams [22]. Concerning progesterone metabolism, P450c17 performance was further characterized by the actual ratio of productive events (androgen formation rate times two, since progesterone has to undergo two catalytic modifications in this case) and abortive events (17α-hydroxyprogesterone formation rate) [22]; in these experiments, androgen formation could also be related to endogenous 17α-hydroxyprogesterone concentrations. In the dual-substrate experiments, ^{3}H/^{14}C ratios were calculated for both the 17α-hydroxyprogesterone and androgen fractions to obtain insight into the rate of approaching equilibrium

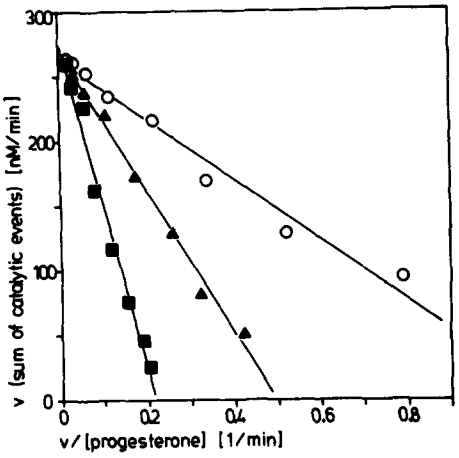

Fig. 1. Eadie–Hofstee plot showing the dependence of the rates of total catalytic events of cytochrome P450c17 in testicular microsomes on varying concentrations of exogenously supplied progesterone without (○), with 0.5 μM (▲) or with 2.0 μM (■) ketoconazole. The straight lines are calculated by linear regression and represent the "combined exogenous hydroxylase plus endogenous lyase activity".

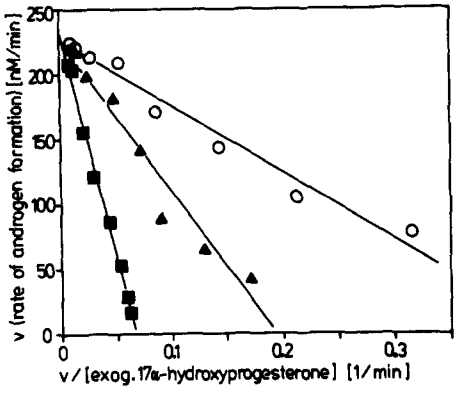

Fig. 2. Eadie–Hofstee plot showing the dependence of the rates of catalytic events of cytochrome P450c17 in testicular microsomes on varying concentrations of exogenously supplied 17α-hydroxyprogesterone without (○), with 0.5 μM (▲) or with 2.0 μM (■) ketoconazole. The straight lines are calculated by linear regression and represent the "exogenous lyase activity".

between exogenous and endogenous 17α-hydroxyprogesterone pools and the priority of both pools with respect to androgen formation. All data are the means of duplicate analyses from two independent incubation assays.

RESULTS

Under initial rate conditions, it becomes evident that ketoconazole competitively inhibits [^{3}H]progesterone turnover by rat testicular P450c17 when its activity is expressed as the sum of catalytic events. Maximal rates V are not influenced (mean for the three series: 273 nm/min, corresponding to a molecular catalytic activity of 5.8/min), but K_M

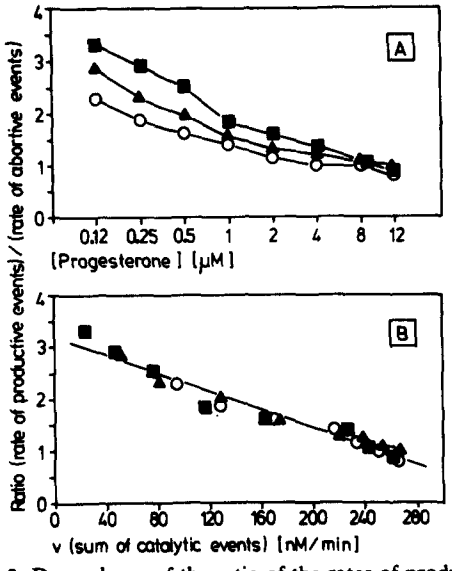

Fig. 3. Dependence of the ratio of the rates of productive vs abortive catalytic events of cytochrome P450c17 in testicular microsomes on (A) varying progesterone concentrations or (B) total rates of catalytic events with progesterone as the substrate without (○), with 0.5 μM (▲) or with 2.0 μM (■) ketoconazole. Data calculated from the same experiment as in Fig. 1.

values for progesterone increase from 233 ± 15 (mean ± SD) to 551 ± 20 and 1300 ± 72 nM in the presence of 0.5 and 2 μM ketoconazole, respectively, resulting in K_I values for ketoconazole of 365 ± 38 and 436 ± 41 nM (Fig. 1). Similarly, the turnover of 17α-hydroxy[^{14}C]progesterone as the exogenous

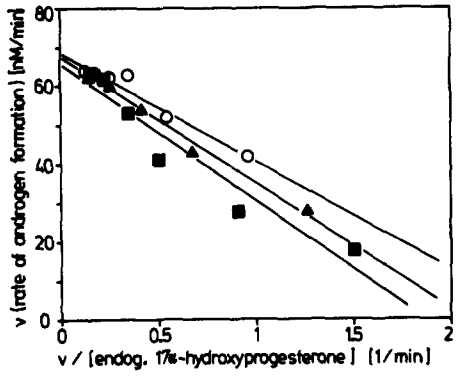

Fig. 4. Eadie–Hofstee plot showing the dependence of the rates of total catalytic events of cytochrome P450c17 in testicular microsomes on varying concentrations of endogenously produced (i.e. with progesterone as the exogenous substrate) 17α-hydroxyprogesterone without (○), with 0.5 μM (▲) or with 2.0 μM (■) ketoconazole. The straight lines are calculated by linear regression and represent the "endogenous lyase activity". Data are calculated from the same experiment as in Fig. 1; endogenous (or intermediary) 17α-hydroxyprogesterone concentrations are those which have been accumulated during the first half of the linear phases of progesterone turnover.

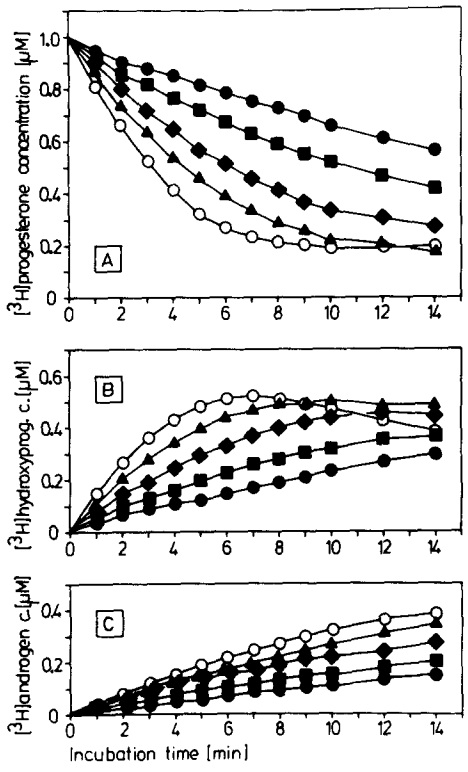

Fig. 5. Metabolism of 1 μM [^{3}H]progesterone (A) towards 17α-hydroxyprogesterone (B) and androgens (androstenedione plus testosterone, C) by cytochrome P450c17 in testicular microsomes in the dual-label/dual-substrate experiment, including simultaneously 1 μM 17α-hydroxy[^{14}C]progesterone (see Fig. 6) without (○), with 0.5 μM (▲), with 1.0 μM (◆), with 2.0 μm (■) or with 4.0 μM (●) ketoconazole.

substrate is also competitively inhibited by ketoconazole: maximal rates V remain again constant (230 nM/min), and K_M values for 17α-hydroxyprogesterone increase from 510 ± 39 to 1180 ± 113 and 3540 ± 144 nM in the presence of 0.5 and 2 μM ketoconazole, respectively, resulting in K_I values of 383 ± 75 and 337 ± 31 nM (Fig. 2).

The refined analysis of P450c17-catalysed progesterone turnover reveals that the ratio of the rates of productive/abortive events (as a measure for relative androgen formation efficiency by P450c17 with progesterone as the substrate) decreases continuously with increasing progesterone concentrations. This ratio is increasingly elevated in the presence of increasing ketoconazole concentrations (Fig. 3A). If these ratios of productive/abortive events are plotted against the individual rates for the sum of P450c17 catalytic events, a linear relation is obtained ($r = -0.979$) that is independent of the presence or absence of the imidazole inhibitor (Fig. 3B). This pattern indicates that ketoconazole displaces progesterone from the P450c17 binding site and prevents its hydroxylation, but that it is unable to influence the intrinsic modulation of androgen formation efficiency of P450c17, which is obviously dictated by the actual concentration of the progesterone–P450c17 complex.

The above conclusion is supported by the result of a second re-analysis of the [^{3}H]progesterone turnover experiment: if initial androgen formation rates are related to those 17α-hydroxy[^{3}H]progesterone concentrations which have accumulated after the first half of the linear phase of progesterone metabolism, provisional apparent K_M values for endogenous 17α-hydroxyprogesterone (at least with regard to this stage of progesterone conversion) can be estimated with respect to androgen formation; they amount to 28, 32 and 35 nM without and with 0.5 μM, and with 2 μM ketoconazole, respectively (Fig. 4). This means that endogenously formed 17α-hydroxy[^{3}H]progesterone (at this stage) should have about an 18-fold higher affinity towards P450c17 than exogenous 17α-hydroxy[^{14}C]progesterone, and that androgen formation from "intermediate-like" 17α-hydroxyprogesterone is much less sensitive towards ketoconazole inhibition (mean $K_I = 6.0$ μM) than androgen formation from "substrate-like" 17α-hydroxyprogesterone (mean $K_I = 0.4$ μM).

Additional evidence supporting these findings is provided by the experiment characterizing ketoconazole effects on the simultaneous turnover of 1 μM [^{3}H]progesterone and 1 μM 17α-hydroxy-[^{14}C]progesterone by P450c17 in rat testicular microsomal membranes (Figs 5 and 6). In the presence of increasing ketoconazole concentrations, elimination rates of both steroid substrates are continuously decreased, but this effect is more pronounced for progesterone. For instance, the period required for a 20% elimination from the [^{3}H]-progesterone pool is extended from 1 to 3.5 min by the addition of 2 μM ketoconazole, whereas the period required for a 20% elimination from the exogenous 17α-hydroxy[^{14}C]progesterone pool is only extended from 4 to 10 min (Figs 5A and 6A). Despite this difference, androgen formation rates from [^{3}H]progesterone and 17α-hydroxy[^{14}C]progesterone appear to be similarly affected by ketoconazole (Figs 5C and 6B).

Differential influences of ketoconazole on cytochrome P450c17 enzyme activities become obvious from calculation of ^{3}H/^{14}C ratios in both the 17α-hydroxyprogesterone and androgen fractions. This transformation allows a comparison of time-dependent changes of ^{3}H-labelled and ^{14}C-labelled steroid pool sizes and therefore of androgen production rates from exogenously added (^{14}C-labelled) and from endogenously formed (^{3}H-labelled) 17α-hydroxyprogesterone. Due to simultaneous inhibition of 17α-hydroxy[^{3}H]progesterone formation from progesterone and of 17α-hydroxy[^{14}C]progesterone turnover by ketoconazole, ^{3}H/^{14}C ratios in the 17α-hydroxyprogesterone fraction are uniformly lower in the presence of the imidazole compound than in control incubations (Fig. 7A). During the initial phases of the incubations, ^{3}H/^{14}C ratios in the androgen fractions are always higher than the corresponding ratios in the 17α-hydroxyprogesterone fractions; this finding corresponds to the previously established concept that the endogenous 17α-hydroxyprogesterone pool is preferentially metabolized over the exogenous one as long as both pools have not reached equilibrium. Unlike the ^{3}H/^{14}C ratios in the 17α-hydroxy-

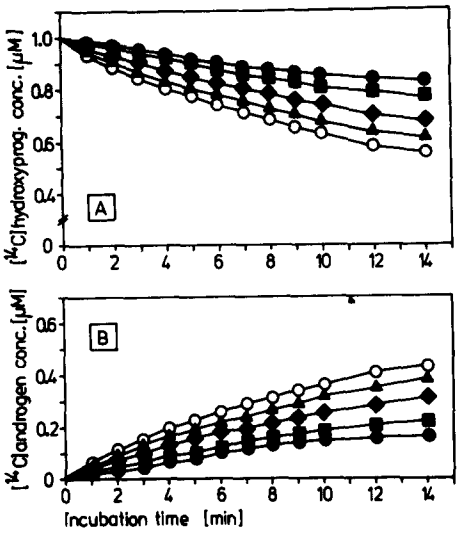

Fig. 6. Metabolism of 1 μM 17α-hydroxy[^{14}C]progesterone (A) towards androgens (androstenedione plus testosterone, B) by cytochrome P450c17 in testicular microsomes in the dual-label/dual-substrate experiment, including simultaneously 1 μM [^{3}H]progesterone (see Fig. 5) without (○), with 0.5 μM (▲), with 1.0 μM (◆), with 2.0 μM (■) or with 4.0 μM (●) ketoconazole.

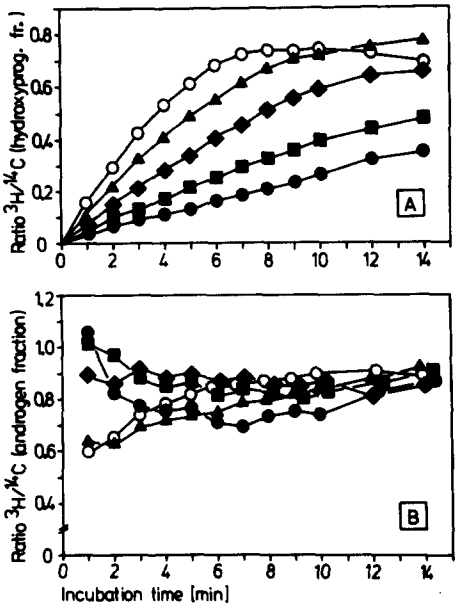

Fig. 7. Changes with time of ^{3}H/^{14}C ratios in the 17α-hydroxyprogesterone (A) and androgen (B) fractions during simultaneous metabolism of 1 μM [^{3}H]progesterone and 1 μM 17α-hydroxy[^{14}C]progesterone by cytochrome P450c17 in testicular microsomes without (○), with 0.5 μM (▲), with 1.0 μM (◆), with 2.0 μM (■) or with 4.0 μM (●) ketoconazole. Ratios are calculated from results depicted in Figs 5 and 6.

progesterone fractions, the ^{3}H/^{14}C ratios in the androgen fractions are even increased in the presence of increasing ketoconazole concentrations (Fig. 7B). With 2 or 4 μM ketoconazole, the initial ^{3}H/^{14}C ratio in the androgen fraction even exceeds 1, i.e. slightly more androgen is formed from [^{3}H]progesterone than from 17α-hydroxy[^{14}C]progesterone. This pattern results in distinctly higher quotients of the ^{3}H/^{14}C ratios in the androgen versus 17α-hydroxyprogesterone fractions {[^{3}H/^{14}C(A)]/[^{3}H/^{14}C(H)]} in the presence of ketoconazole (data not shown). These data describing imidazole inhibitor effects on differential label distribution in intermediate and final product fractions are transformed to allow direct comparison of the pool sizes and the respective elimination rates at a single time-point in Fig. 8. If, for instance, the ratio of elimination rates from the endogenous versus the exogenous 17α-hydroxyprogesterone pools is divided by the ratio of the endogenous versus the exogenous 17α-hydroxyprogesterone pool sizes, a quotient is obtained that amounts to 4.0 under control conditions, but is increased to values of 6.4, 11.6, 17.6 and 21.2 in the presence of 0.5, 1, 2 and 4 μM ketoconazole, respectively. This result proves that ketoconazole enhances the relative preference with which the endogenous 17α-hydroxyprogesterone pool is metabolized as compared to the exogenous one.

DISCUSSION

Cytochrome P450c17 from rat testis has been characterized as an enzyme with a bifunctional catalytic site [22]; this implies that in contrast to other multifunctional enzymes (multienzymes or multienzyme polypeptides) or multienzyme complexes [33, 34], the intermediate, 17α-hydroxyprogesterone, is subjected to "functional" rather than "spatial" channelling since only marginal structural rearrangements have to occur between the first and the second catalytic event [22]. Cytochrome P450c17 is strongly associated with the membranes of the smooth endoplasmic reticulum in steroidogenic tissues, and hydrophobic steroid substrates and imidazole-type inhibitors probably reach the active site from within the membrane [35–37]. Recent calculations have suggested that the main fraction of the intermediary 17α-hydroxyprogesterone associated with the membrane compartment is in fact bound to the P450c17 enzyme [38]. However, any relation of P450c17 function to tertiary structure is impossible so far, though some provisional assumptions can be made on the basis of sequence alignments with P450cam (CYP101) [24]. The use of inhibitors has been suggested to be useful in the elucidation of the functional coupling of both P450c17 activities [22], and this strategy is applied in the present study.

Ketoconazole is a competitive inhibitor of both the P450c17-dependent hydroxylase and lyase activities [3, 4, 7, 9–11, 13, 18]. This is confirmed by this study (Figs 1 and 2). Inhibition constants K_I or IC_{50} values for ketoconazole with respect to P450c17 inhibition as reported in the literature vary considerably within the 10 nM to μM range [4, 10, 11, 13, 14, 18], but these differences may simply arise from different enzyme concentrations used [37], different enzyme sources (species and

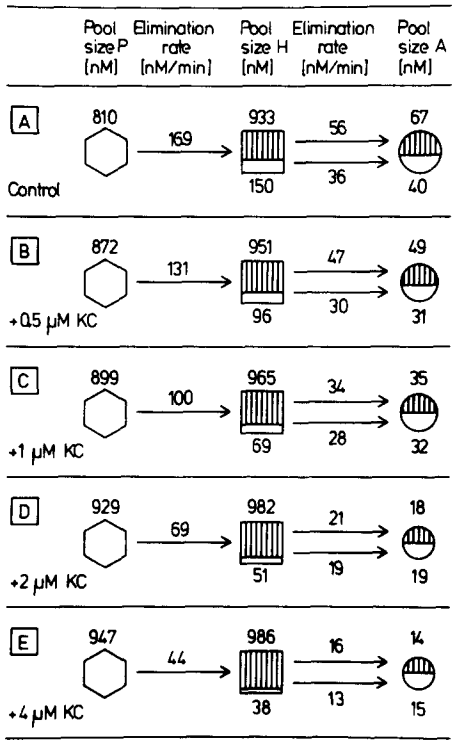

Fig. 8. Comparison of the sizes of ^{3}H-labelled (white areas) and ^{14}C-labelled (hatched areas) pools of progesterone (P), 17α-hydroxyprogesterone (H) and androgens (A) with the corresponding elimination rates during an early stage (1 min) of simultaneous metabolism of 1 μM [^{3}H]-progesterone and 1 μM 17α-hydroxy[^{14}C]progesterone by cytochrome P450c17 in testicular microsomes without (A), with 0.5 μM (B), with 1.0 μM (C), with 2.0 μM (D) or with 4.0 μM (E) ketoconazole.

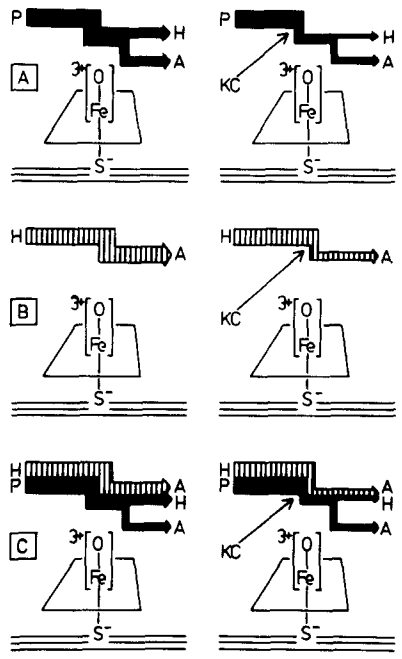

Fig. 9. Modelling of steroid flux rates through cytochrome P450c17-catalysed reactions with progesterone (P) alone (A), with 17α-hydroxyprogesterone (H) alone (B), or with both steroids present simultaneously (C), in the absence (left-hand diagrams) or presence (right-hand diagrams) of ketoconazole. The first oxygen insertion step but not the intermediate processing step is affected by ketoconazole (see text).

organ) or different substrates used (5-ene versus 4-ene steroids). In contrast to all the previous reports, however, the present study for the first time takes into account a possible effect of ketoconazole on the "handling" of intermediary, endogenously formed 17α-hydroxyprogesterone by P450c17.

Modulation of the ratio of the rates of productive/abortive P450c17 catalytic events (Fig. 3) indicates that ketoconazole competes with the substrate, progesterone, for access to and/or binding within the active site and prevents its hydroxylation (first P450c17 catalytic event) by the P450-activated oxygen species. Since imidazoles, in contrast to steroid substrates, interact directly with the heme [39], disruption of oxygen activation by ketoconazole seems also possible. However, once hydroxylated, the intermediate (17α-hydroxyprogesterone or a labile analogue) is retained at the active site (which is, at this time, blocked by the steroid and thus either not accessible for and/or not modifiable by ketoconazole) and processed to the androgen with a probability which is dictated by the ligand–enzyme complex concentration [22] and possibly also by the rate of supply of electrons for oxygen reduction [25]. This model is schematically depicted in Fig. 9A. It should be emphasized here that this model does not mean that ketoconazole is unable to inhibit androgen production from progesterone through P450c17 catalysis. Undoubtedly, it does so; but its action is, as concluded from the data reported here, at the first oxygen insertion step and obviously not at the stage of intermediate processing.

The above conclusion is not contradictory to the fact that ketoconazole inhibits P450c17 lyase activity acting on exogenous 17α-hydroxyprogesterone as substrate (Figs 2 and 9B). Its conversion to androgen corresponds, when considered in terms of ligand binding and ligand–oxygen interaction modes, to a first oxygen activation and insertion event, so that entrance of 17α-hydroxyprogesterone into the active site and first-step monooxygenation (resulting in C—C bond cleavage) can be compared with that of progesterone. We propose that the well-accepted "bifunctionality" of the P450c17 enzyme [22, 24–26] may be understood in terms of contrasting first versus second catalytic events rather than by contrasting hydroxylase versus lyase activites.

Provisional estimates for affinities of endogenously formed 17α-hydroxyprogesterone to P450c17 (Fig. 4) indicate about an 18-fold higher affinity of intermediary compared to substrate-type 17α-hydroxyprogesterone, at least at the stage investigated here. This value is in good agreement with previously found differences in the efficiency of exogenous versus endogenous steroid turnover in the course of testicular androgen biosynthesis under steady-state conditions [40].

Comparison of the time-courses of androgen formation from endogenous and exogenous 17α-hydroxyprogesterone within the dual-label/dual-substrate experiment provides final evidence that the processing of the enzyme-bound intermediate is insensitive towards ketoconazole inhibition (Figs 8A–E and 9C). For example, at an early stage of the control incubation experiment, the total 17α-hydroxyprogesterone pool amounts to 1080 nM, and the elimination rate from this pool amounts to 92 nM/min. Subdivision of this value into elimination rates from the ^{3}H-labelled ("endogenous") 17α-hydroxyprogesterone pool on the one hand (36 nM/min corresponding to 150 nM pool size) and from the ^{14}C-labelled ("exogenous") 17α-hydroxyprogesterone pool on the other hand (56 nM/min corresponding to 930 nM pool size) reveals that the endogenous pool is metabolized at a 4.0-fold higher relative rate than the exogenous one (Fig. 8A). In agreement with previous results [29, 38], the accumulation of endogenous 17α-hydroxyprogesterone in the P450c17-accessible membrane domain and at the P450c17 active site itself should therefore be 4-fold higher than the parallel accumulation of exogenous 17α-hydroxyprogesterone.

If the same calculation is done with elimination rates from the 17α-hydroxyprogesterone pools in the presence of imidazole inhibitor at an identical stage of steroid metabolism, it is found that in the presence of 0.5, 1, 2 and 4 μM ketoconazole, the ratio of the P450c17-associated fraction of the intermediary 17α-hydroxyprogesterone versus the P450c17-associated fraction of the exogenous 17α-hydroxyprogesterone increases by factors of 1.6, 2.9, 4.4 and 5.3, respectively, as compared to controls (Fig. 8A–E). Thus, ketoconazole shifts the preference of intermediate-type over substrate-type 17α-hydroxyprogesterone conversion by P450c17 in favour of the former.

In conclusion, this study provides new insights into the function of the steroidogenic cytochrome P450c17. This has previously been established as a specialized model system for studying direct transfer ("channeling") of intermediates within enzymes [22, 24, 25, 28, 29, 34, 41, 42]. The present study reveals that ketoconazole as a paradigm of an imidazole-type inhibitor of P450c17 interacts with the binding of steroid substrates (whether progesterone or 17α-hydroxyprogesterone) and inhibits oxygen insertion (resulting in either 17α-hydroxylation or C17–C20 cleavage), but does not alter the efficiency of intermediary 17α-hydroxyprogesterone channelling. This conclusion may be a useful basis for further research on cytochrome P450 structure–function relationships, especially since a parallel pattern has been suggested in the case of the P450-14DM, where the final oxidative decarbonylation step of the lanosterol 14α-methyl demethylase reaction sequence appears to be insensitive towards ketoconazole, too [6].

The need for specific inhibitors of P450c17-dependent steroid-17,20-lyase activity in order to lower androgen secretion, for instance in patients with androgen-dependent malignomas, has been emphasized [3, 4, 19, 20, 24, 43]. Though the present study confirms previous reports that androgen formation from pregnenolone or progesterone is inhibited by ketoconazole, it also elucidates, that the inhibition of lyase activity with exogenous 17α-hydroxyprogesterone as the substrate does not necessarily implicate parallel inhibition of endogenous lyase activity. If this interpretation of ketoconazole action on the bifunctional cytochrome P450c17 represents a general mechanism [6], the design of specific lyase inhibitors [24] should be expected to be more difficult than the design of imidazole ligands that take advantage of structural and functional differences between the adrenal and testicular P450c17 species [4, 25, 26].

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