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A Microcompartmentation Analysis of Intermediate Leakage Response to Substrate Excess in a Membrane-Bound Bifunctional Enzyme: Local Control of Hydroxyprogesterone Channeling Efficiency During Cytochrome P450XVII-Catalysed Androgen Biosynthesis

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Evidence is presented for the first time that the cytochrome P450XVII-catalysed androgen formation from progesterone (P) in rat testicular microsomal membranes represents a metabolic sequence that exhibits the ability of intrinsic regulation of intermediate transfer and product formation efficiency. Exposure of this system, which catalyses a hydroxylation and oxidative cleavage reaction sequence, to increasing P concentration results in a decreased specific retention of the putative intermediate, 17α-hydroxyprogesterone (HP) in the membrane compartment, and in a decreased HP conversion to androgens in favour of increasing HP transfer into the extramembrane space. This behaviour results in a decreased ratio of product vs. intermediate formation rates, which is interpreted as a partial "uncoupling" of the normal hydroxylation and cleavage reaction sequence catalysed by P450XVII. A similar pattern can likewise be observed in isolated testicular Leydig cells after exposure to increasing P concentrations under more physiological continuous-flow conditions. Further calculations indirectly indicate that the specific retention of HP in the membrane compartment can partially be attributed to its specific association with the P450XVII during catalysis. The results strongly suggest the existence of a local "channel" that becomes more leaky and therefore less effective if loaded with high influx rates. This pattern may be related to significant but incomplete competition of exogenously entering P and endogenously formed and transiently bound HP for oxygen attack at the P450XVII active site.

Key words: androgen biosynthesis, channeling, compartmentation, cytochrome P450, metabolism, testis

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The metabolic pathways of sex steroid hormone biosynthesis and metabolism in the gonads and other steroidogenic tissues are bound to intracellular membrane compartments [1–5], which generate the specific environments determining enzyme-substrate and enzyme-enzyme interactions [3–11]. With the exception of the cholesterol side-chain cleavage reaction, which takes place in the inner mitochondrial membranes, further metabolism of its product, pregnenolone, to androgens and estrogens occurs in the membranes of the smooth endoplasmic reticulum (Fig. 1), which are able to specifically concentrate pregnenolone to a high degree [1,3,4].

The key enzymes in this reaction sequence and the most sensitive targets to hormonal regulation [12–17] are highly specialized monooxygenase systems. These are functional assemblies of a flavoprotein as the hydrogen acceptor and primary electron carrier, an iron-sulfur protein as the second electron carrier (only in mitochondrial systems), and a cytochrome P450 that functions as the active site with regard to both oxygen activation and substrate binding and turnover [1,2]. The sequence of substrate binding, first electron transfer, oxygen binding, second electron transfer, oxygen cleavage, oxygen insertion, and product dissociation is generally referred to as the catalytic cycle of cytochromes P450 [1,2,18,19]. The steroidogenic P450s consist of one polypeptide chain and one heme moiety per molecule as other P450s [20–23], but are unique in catalysing subsequent coordinated hydroxylation *and* carbon-carbon bond scission reactions [1,2]: The cholesterol side-chain cleavage system (P450XI; EC 1.14.15.6) catalyses two hydroxylation and one oxidative cleavage reaction on the cholesterol molecule [18,24]; the steroid-17α-monooxygenase/C17,20-lyase system (P450XVII; EC 1.14.99.9/4.1.2.30) catalyses one hydroxylation and one oxidative cleavage reactions on the progesterone molecule [17,20,21,23,25]; and the aromatase system (P450XIX) catalyses two hydroxylation and one oxidative cleavage reaction on the androstenedione (or testosterone) molecule [26–28]. Though it is most likely that steroidogenic P450s accept their substrates from within the membrane lipid phase [3,5,29–31], it is not yet clear if the subsequent coordinated reactions occur at identical sites of the respective P450s or if there are conformational changes of the enzyme proteins or small spatial shifts of the intermediates relative to the peptide chain during the reaction sequence [8,10,18,24,32].

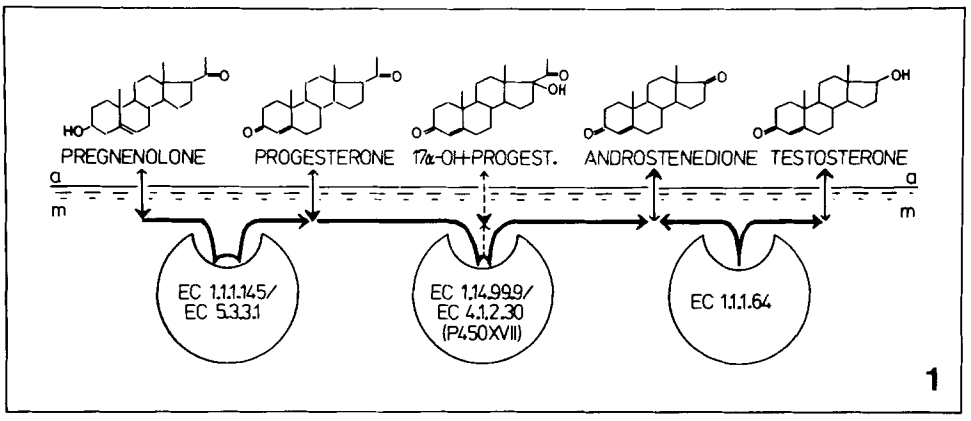


Fig. 1. Survey of reaction sequences involved in androgen formation from pregnenolone in smooth endoplasmic reticulum membranes from rat testis (m, membrane compartment; a, aqueous compartment).

The hydroxylated intermediates of the steroidogenic P450-catalysed concerted hydroxylation and cleavage reactions are mainly present in an enzyme-bound state and specifically retained in the membrane compartments though some leakage from the enzyme and the membrane phase seems to be possible [5,18,24,33–36]. The degree of such intermediate leakage is much higher in the P450XVII system than in the P450XI or P450XIX systems [2,5,28,34,36] but has rarely been subjected to a complete quantitative analysis.

Steroidogenic P450s may therefore be regarded as bifunctional (P450XVII) or trifunctional (P450XI and P450XIX) enzymes, and the reaction sequences may be interpreted as special cases of interacting catalytic systems [37], where the two or three catalytic cycles of the complete reaction can probably occur at one site near the heme of one P450 protein. In this context, P450s may represent suitable models to study possible intrinsic control [38,39] of "unproductive" intermediate leakage vs. "productive" intermediate channeling resulting in efficient product formation. On the basis of these considerations, the present study tests the hypothesis that increasing concentrations of progesterone, the natural substrate of P450XVII in rat testis microsomal membranes, will result in over-proportional formation of the intermediate, 17α-hydroxyprogesterone, with the consequence of decreased androgen formation efficiency. It further investigates whether substrate excess affects the retention of intermediary 17α-hydroxyprogesterone in the membrane compartment. In relation to recent concepts of intermediate handling by the hydrophobic cores of multienzyme complexes or multifunctional enzymes [9,37,40–42], the results allow the introduction of the model of substrate-dependent, intrinsic control of the leakiness of the local metabolic channel and intermediate transfer efficiency at the P450XVII active site within the androgen biosynthetic pathway. This may provide a generally new aspect of metabolic control strategy in hormone-synthesizing systems [9,38,43].

METHODS

The basic methodological design of this study was essentially that described previously [5]. Briefly, microsomes were prepared from testes of postpubertal Han: Wistar rats by homogenization and repeated differential centrifugation. The final 6.8 $\times 10^6$g $\times$min pellet was essentially free from mitochondrial contamination as assessed by glutamate dehydrogenase (EC 1.4.1.2) and cholesterol side-chain cleavage activities. The incubation assays contained microsomal suspensions in 50 mM phosphate buffer (pH 7.4) corresponding to 0.58 mg protein/ml [44], 0.23 mg lipid/ml [45], and 55 nM cytochrome P450 [46], as well as 130 μM NADPH and 280 μM $MgCl_2$. The reaction (32°C incubation temperature) was started by addition of progesterone (including 500 Bq of [^{3}H]progesterone/ml) to final concentrations of 0.3 μM, 1.0 μM, 3.0 μM, or 10 μM, respectively. After the incubation times specified in the Results section, aliquots were transferred to ice-cooled tubes containing a 32-fold excess of $NADP^+$ to stop completely further metabolism [5]. Steroids from these original suspensions or from the aqueous supernatants obtained after centrifugation (6.8×10^6g $\times$ min) were extracted with diethylether, and the progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone fractions were separated by thin-layer chromatography [47]. Androstenedione and testosterone fractions were combined to the androgen fraction since both steroids are in a $NADPH/NADP^+$-controlled equilibrium [48]. Radioactivity in the microsomal suspensions and the corresponding supernatants, as well as in the individual steroid

fractions allowed the calculation of time-dependent steroid metabolism (Fig. 2) and steroid distribution between buffer and membrane compartment in terms of the partition coefficient K_P (Fig. 3). From these data, net transfer rates from the membrane-associated, intermediary 17α-hydroxyprogesterone pool to the aqueous compartment (as the measure of intermediate leakage) and to the androgen fraction (as the measure of intermediate channeling) could be calculated (Fig. 4). Furthermore, the dependence on initial progesterone concentrations of the ratio of P450XVII-catalysed hydroxylation events (1st catalytic cycle: sum of 17α-hydroxyprogesterone and androgen formation rates) vs. P450XVII-catalysed cleavage events (2nd catalytic cycle: androgen formation rate) [5,18] was determined (Fig. 5). Finally, the knowledge of both the local concentrations of progesterone and 17α-hydroxyprogesterone in the membrane compartment (Figs. 2 and 3) and of P450 * progesterone and P450 * 17α-hydroxyprogesterone dissociation constants corrected for hydrophobicity [49] allow to clarify whether the expected linear correlations [50] between the measured enzymatic activities of P450XVII and the calculated concentrations of P450 * progesterone and P450 * 17α-hydroxyprogesterone complexes do exist (see Fig. 8).

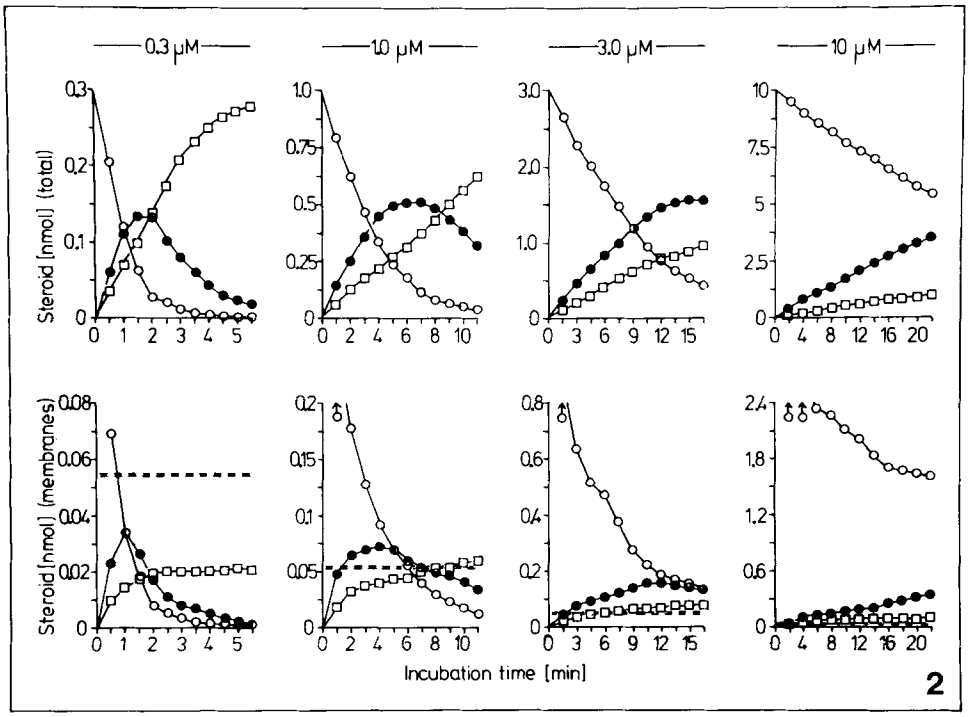


Fig. 2. Changes with time of [^{3}H]progesterone (open circles), 17α-hydroxy[^{3}H]progesterone (filled circles), and [^{3}H]androgen (open squares) amounts (related to 1 ml of incubation volume) in the whole incubation assay (upper diagrams) and in the membrane phase (lower diagrams) during P450XVII-catalysed metabolism of increasing progesterone concentrations (0.3–10 μM) in rat testicular microsomal membranes (duplicate analyses from n = 2 incubation experiments). The broken lines in the lower diagrams represent the total concentration of P450.

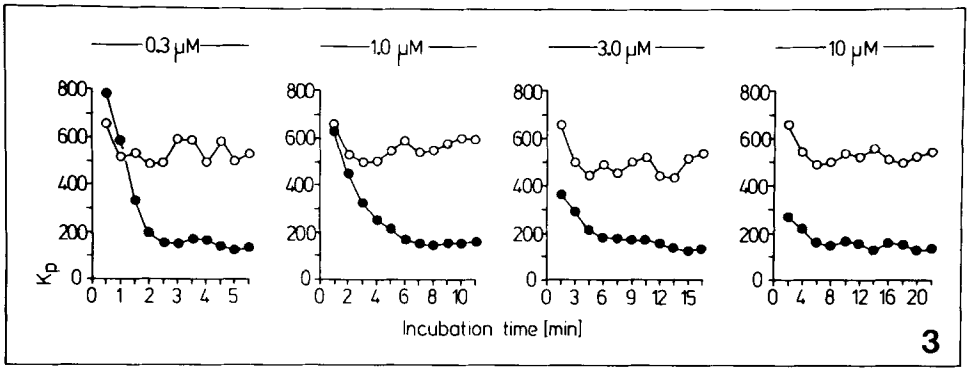


Fig. 3. Partition coefficients K_p for progesterone (open circles) and 17α-hydroxyprogesterone (filled circles) distribution between membrane and aqueous compartments during P450XVII-catalysed metabolism of increasing progesterone concentrations (0.3–10 μM) in rat testicular microsomal membranes. Values derived from data presented in Figure 2.

RESULTS

The time-dependent changes of progesterone, 17α-hydroxyprogesterone, and androgen concentrations in the whole incubation assay as well as in the microsomal membrane compartment are documented in Figure 2. With increasing substrate (progesterone) concentrations, the P450XVII-catalysed progesterone metabolism shifts from exponential to linear time-decay. During the initial phase of the incubations, the putative intermediate (17α-hydroxyprogesterone) and the products (androgens) accumulate simultaneously. The primary data presented in Figure 2 indicate that the ratio of initial 17α-hydroxyprogesterone vs. androgen formation rates increases with elevated initial progesterone concentration. Time-dependent 17α-hydroxyprogesterone concentration changes obviously reflect simultaneous invasion and elimination kinetics in terms of Bateman functions, which generally describe concentration-time curves of the intermediate in any sequence of two irreversible first-order processes [51]. It is an interesting and

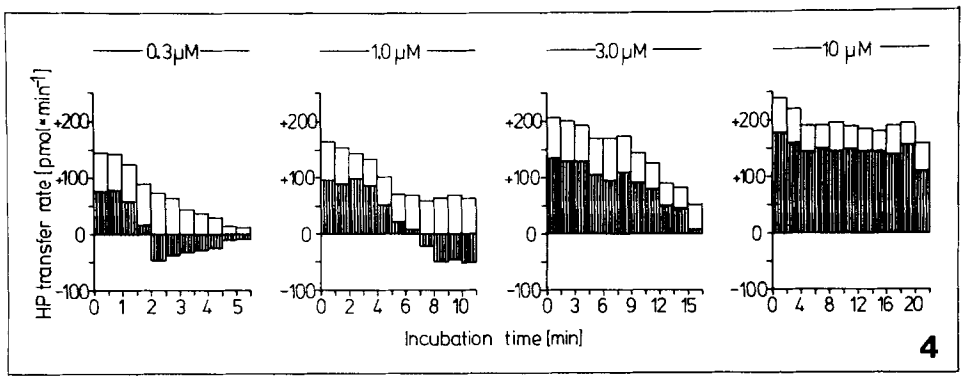


Fig. 4. Net transfer rates of intermediary 17α-hydroxyprogesterone (HP) from the membrane compartment into the buffer compartment (hatched areas) and from the membrane compartment into the androgen fraction (white areas) during P450XVII-catalysed metabolism of increasing progesterone concentrations (0.3–10 μM) in rat testicular microsomal membranes. Values derived from data presented in Figure 2.

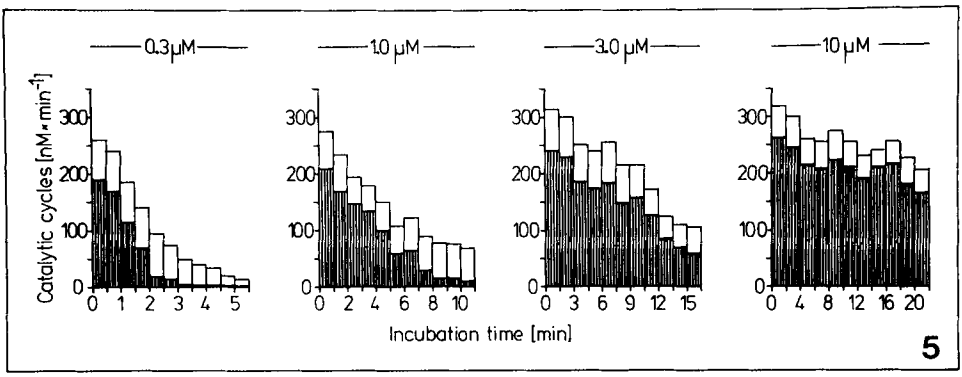


Fig. 5. Hydroxylation events (1st catalytic cycle of P450XVII, hatched areas) and cleavage events (2nd catalytic cycle of P450XVII, white areas) during P450XVII-catalysed metabolism of increasing progesterone concentrations (0.3–10 µM) in rat testicular microsomal membranes. Values derived from data presented in Figure 2.

general phenomenon that the maxima of these curves are shifted to shorter times in the membrane compartment as compared to the whole incubation assay (Fig. 2), indicating that the ratios of invasion vs. elimination constants for the 17α-hydroxyprogesterone pool are higher in the membrane compartment than in the whole incubation assay.

This pattern can be explained by specific accumulation of 17α-hydroxyprogesterone in the membrane compartment during the initial phase of the reaction as revealed by the partition coefficients K_P (Fig. 3). The most interesting observation of this study is that the specific initial retention of the putative reaction intermediate within the membrane pool continuously decreases in response to elevated initial substrate concentrations, whereas an equilibrium of 17α-hydroxyprogesterone partition ($K_P \approx 150$) is reached in any case at longer incubation periods (Fig. 3). The ratio of net transfer rates of membrane-associated 17α-hydroxyprogesterone to the buffer compartment vs. transfer to the androgen fraction increases in response to elevated substrate concentration (Fig. 4). Similarly, the ratio of P450XVII-catalysed hydroxylation vs. cleavage events during the initial phase of the reaction changes in favour of hydroxylation (Fig. 5) resulting in a decreasing ratio of product vs. intermediate formation rates (Fig. 2) in response to elevated progesterone concentrations.

Thus, exposure of rat testicular microsomal suspensions to increasing progesterone concentrations in vitro results in a diminution of specific retention of 17α-hydroxyprogesterone by the membrane compartment, in an increased leakage of 17α-hydroxyprogesterone relative to androgen formation, and therefore to a decreased product formation efficiency. This pattern indicates a less efficient intermediate transfer (Fig. 4) and an increasing "uncoupling" of the hydroxylation and cleavage reactions catalysed by microsomal P450XVII (Fig. 5) in response to elevated substrate concentration.

DISCUSSION

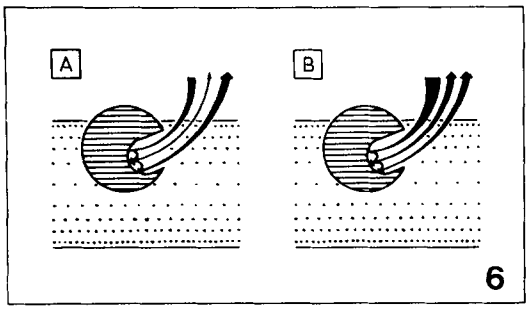
There is increasing evidence that the appearance of free intermediates in certain metabolic pathways, which may be understood as organized clusters of interacting enzyme proteins [40–43,52,53] catalysing a coordinated reaction sequence in which

intermediates can be transferred without transitory solvation, could be considered as the consequence of partial leakage and stabilization of the enzyme-bound intermediates from the hydrophobic cores of such aggregates [24,42]. In some systems, the accumulation of intermediates has been proposed to depend on the degree of multienzyme complex organization and the actual metabolic situation [8,9,37,41,42,54]. As to the nature of intermediates in sex steroid biosynthetic processes, the possible alternatives, whether intermediates are truly enzyme-bound, whether they are by-products of transient, labile enzyme-bound states, or whether they represent leaky pathways resulting from steroid transfer between active centres of enzymes, are controversely discussed [5,18,24,27,28,33–36,55]. Some clarification has emerged from a recent study [5] showing that 17α-hydroxyprogesterone, the intermediate within the P450XVII-catalysed progesterone to androstenedione conversion, can be specifically retained in endoplasmic reticulum membranes if being in the transient state, but that it is likewise able to leave and to re-enter the membrane-associated intermediary 17α-hydroxyprogesterone pool. Only the latter pool has been proposed to be directly accessible to P450XVII and to determine androgen production [5]. This conclusion is confirmed in the present study: The pharmacokinetic law [56], that the maximum of the concentration-time curve of a truly intermediary compartment must lie on the elimination curve of the substrate compartment, is roughly fulfilled for the membrane-associated 17α-hydroxyprogesterone pool but not in the case of the total 17α-hydroxyprogesterone pool (Fig. 2).

The novel evidence coming from the results of the present study is that within the P450XVII system in testicular microsomal membranes, increasing substrate concentrations lead to decreased specific retention of the intermediate in the membrane compartment (Fig. 3), to decreased ratio of hydroxyprogesterone transfer to the androgen pool vs. hydroxyprogesterone transfer into the aqueous compartment (Fig. 4), and to increased ratio of hydroxylation (1st catalytic cycle) events vs. cleavage (2nd catalytic cycle) events (Fig. 5). Substrate excess therefore results in overproportional formation and subsequent expulsion of the intermediate at the cost of product formation efficiency (Fig. 6).

In the stationary model used in this study, the intermediate spill-over at high progesterone concentration is of a "reversible" nature, since 17α-hydroxyprogesterone can be reutilized by the lyase function of P450XVII when progesterone concentrations decrease (Figs. 2 and 4). Reanalysis of data previously published in a different context [57], however, reveals that under continuous-flow conditions [58], which more closely resemble the physiological state [34,35], intermediate leakage response to increasing progesterone concentration is obviously of an "irreversible" nature. Continuous exposure of isolated and purified Leydig cells from rat testes to increasing progesterone concentrations results again in an increased ratio of hydroxylation events (1st catalytic cycle of P450XVII) vs. cleavage (2nd catalytic cycle) events (from 1.45 with 0.05 μM progesterone to 2.33 with 2.0 μM progesterone) and thus to an "irreversible" intermediate spill-over into the extracellular milieu (Fig. 7).

The intermediate-channeling vs. spill-over phenomenon, which has been intensively dealt with theoretically, and which is strongly supported by the existence of specific enzyme interactions in certain metabolic sequences [9,24,37,42,52,54,59], has been proved, for example, for the glycolytic pathway with isolated enzymes [53] as well as with whole cells [60], for the tryptophan synthase [61,62], and, more preliminarily, for mitochondrial β-oxidation [63], and, in analogy, for the coupled dopamine transport-



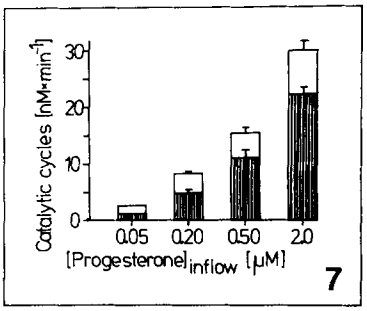


Fig. 6. **Left:** Proposed model of intermediate leakage from P450XVII (shaded) in rat testicular microsomal membranes (dotted area) upon exposure to low (scheme A) or high (scheme B) substrate concentration. Excess substrate increases the ratio of intermediate/product release.
Fig. 7. **Right:** Hydroxylation events (1st catalytic cycle of P450XVII, hatched areas) and cleavage events (2nd catalytic cycle of P450XVII, white areas) during P450XVII-catalysed metabolism of increasing progesterone concentrations (0.05–2.0 μM) by 10^6 isolated purified Leydig cells from rat testes under steady-state conditions in a cell-perifusion system. Means ± SEM (n = 4) from data presented in [57].

dopamine monooxygenase system [64]. It must be emphasized, however, that the systems tested so far differ in at least two respects from the P450 system investigated in the present study. First, *one* active site of P450XVII is able to catalyse both the hydroxylation and cleavage reactions with progesterone as the substrate [1,17,18,20,21,23–25,32], whereas tryptophan synthase, for example, catalyses the two subsequent reactions at *two* spatially distinct and functionally different active sites [62]. The P450XVII-catalysed reaction sequence is also different from some other bifunctional (so-called tandem) enzymes [39]. Second, the common approach to prove intermediate channeling is to measure the effect of *intermediate* addition on the rate of product formation from the substrate [5,55,60,61]; in contrast, the present study for the first time proves the *substrate*-dependence of intermediate transfer efficiency on the basis of a microcompartmentation analysis by showing that high substrate influx into the P450XVII-catalysed reaction sequence reduces specific 17α-hydroxyprogesterone retention in the membrane space and its turnover to androgens.

There may be some possible interpretations for this as yet unknown phenomenon. It could be discussed in terms of classical competition between progesterone and 17α-hydroxyprogesterone for the attack of activated oxygen at the P450XVII [65]. The intermediate leakage response to increasing progesterone concentrations may then be the consequence of the as yet unknown pattern that the feedforward inhibition (by progesterone) of the cleavage of the endogenously formed 17α-hydroxyprogesterone becomes more prominent than the feedback inhibition (by the intermediary hydroxyprogesterone) of the hydroxylation of the exogenously added progesterone [38]. The assumption of limiting P450XVII-lyase activity in relation to the P450XVII-hydroxylase activity, however, cannot sufficiently explain the data, since the lyase activity exceeds the hydroxylase activity in testicular microsomes [20,21] as well as in P450XVIIcDNA-transfected cells [23].

The relevance of the hypothesis, that competition between progesterone and 17α-hydroxyprogesterone may account for intermediate spill-over and reduced product formation after high substrate loading of P450XVII, can be proved, in the first instance, by the demonstration of linear correlation between the measured rate of total catalytic

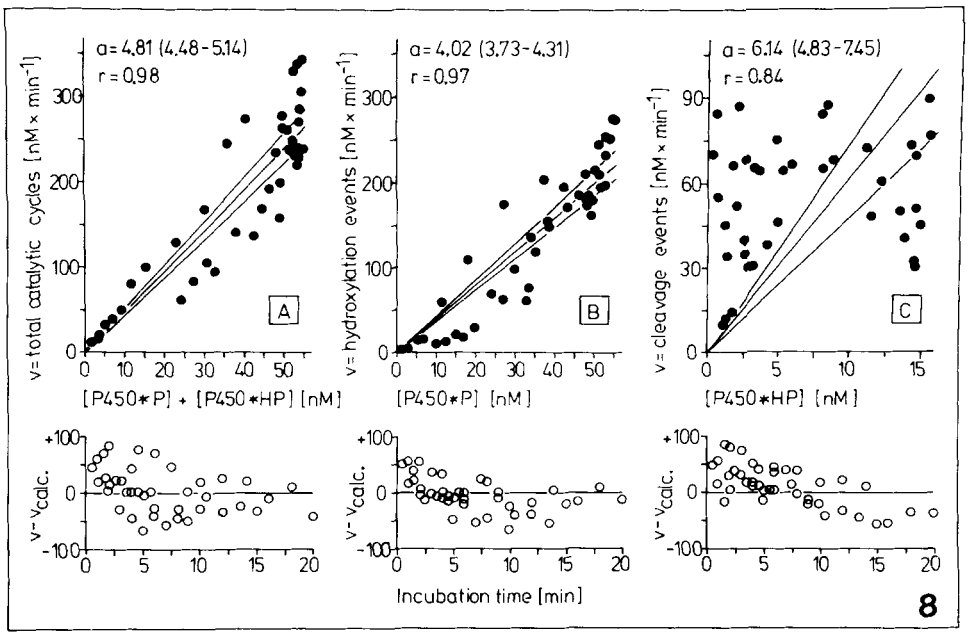


Fig. 8. Dependence of measured catalytic activities of P450XVII (data from Fig. 5) on P450 * substrate complex concentrations calculated from steroid concentrations in the membrane compartment (see text for details). **A:** Total catalytic cycles vs. sum of P450 * progesterone (P) plus P450 * 17α-hydroxyprogesterone (HP) concentrations. **B:** Hydroxylation events (first catalytic cycle) vs. P450 * progesterone concentrations. **C:** Cleavage events (second catalytic cycle) versus P450 * 17α-hydroxyprogesterone concentrations. The respective regression coefficients (slopes a) and the 95% confidence intervals, as well as the correlation coefficients (r) are given. The respective residual plots are shown in the lower diagrams; it should be noted that the *relative* deviations are much greater in plot C than in A or B.

cycles (sum of hydroxylation and cleavage events, see Fig. 5) and the sum of P450 * progesterone complex and P450 * 17α-hydroxyprogesterone complex concentrations (Fig. 8A). The latter can be calculated from the actual progesterone and 17α-hydroxyprogesterone concentrations found in the membrane space (Figs. 2 and 3) and the previously determined dissociation constants related to the membrane compartment for P450 * progesterone (155 μM) and for P450 * 17α-hydroxyprogesterone (319 μM) [49]. The calculated regression coefficient, which is equivalent to the turnover number, is found to be 4.81 nM $\times$(min $\times$ nM)$^{-1}$. Similarly, the observed rates of hydroxylation events show roughly linear dependence on the calculated P450 * progesterone complex concentrations (Fig. 8B). However, this assumption of free steroid distribution and competition within the membrane compartment during P450XVII-catalysed progesterone turnover is totally insufficient in explaining a dependence of cleavage events (2nd catalytic cycle) on P450 * 17α-hydroxyprogesterone concentrations (Fig. 8C), because during the initial phase of the incubations, the P450 * 17α-hydroxyprogesterone concentrations calculated on the basis of that assumption are far too low to account for the observed androgen formation rates. Though it cannot be excluded that methodological limitations may lead to an underestimation of 17α-hydroxyprogesterone retention in the membranes, this observation indicates that the assumption of free competition of

progesterone and 17α-hydroxyprogesterone within the membrane space with respect to P450-binding may be wrong under dynamic conditions.

Thus, it is clearly demonstrated that the determination of steroid concentrations in the relevant membrane compartments represents a substantial advantage in describing intermediate channeling and specific retention, but is still not accurate enough to explain this phenomenon completely. One attractive hypothesis to explain this discrepancy would be that a certain amount, if not all, of the intermediary 17α-hydroxyprogesterone, which is specifically accumulating in the membrane space during the initial phase of the reaction, especially at low substrate concentrations (Fig. 3), is additionally bound to the P450XVII. Another interpretation would be that endogenously formed 17α-hydroxyprogesterone can only partly be displaced by the exogenously added progesterone. Both assumptions implicate the specific though incomplete retention of the intermediate at the active site of P450XVII during catalysis, at least until an equilibrium between endogenously formed and exogenously reentering 17α-hydroxyprogesterone is reached. This conclusion could explain both the specific accumulation of intermediary 17α-hydroxyprogesterone [5] in the membrane compartment (Fig. 3) and the previous observation that the apparent K_M values for the P450XVII-lyase reaction are by a factor 5–6 higher for exogenously added than for endogenously formed 17α-hydroxyprogesterone [57].

Thus, progesterone excess may cause macroscopic uncoupling of the P450XVII-hydroxylase and lyase activities by hindering the intermediate to remain at the catalytic site and to be exposed to the productive second oxygen attack, with the consequence that 17α-hydroxyprogesterone leaves the catalytic site and the membrane compartment. Whether such a substrate-induced partial shift from productive intermediate channeling to unproductive intermediate leakage has any physiological relevance in the control of androgen biosynthesis and possibly other organized metabolic sequences in vivo remains an unsolved problem that is currently under investigation.

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