Distribution of progesterone-binding cytochrome P450 and steroid-17α-hydroxylase/C-17,20-lyase within different compartments of the rat testis

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Progesterone-binding capacity of cytochrome P450 and rate of progesterone consumption by cytochrome P450-dependent hydroxylases are highest in the Leydig cell fraction but are also detectable in the non-Leydig cell fraction of interstitial cells as well as in seminiferous tubules from rat testis. The Leydig cell compartment, however, contributes only to a minor extent to the total progesterone binding and hydroxylation within the whole testis.

Cytochrome P450 *Leydig cell* *Steroid hydroxylase* *Testis*

1. INTRODUCTION

Progesterone is known as one precursor of androgen biosynthesis in rat testicular tissue [1]; formation of androgens is catalyzed by the steroid-17α-hydroxylase (EC 1.14.99.4; product: 17α-hydroxyprogesterone) and the steroid-C-17,20-lyase (EC 4.1.2.30; product: androstenedione) [2–4]. Both enzymes are associated with the microsomal fraction of rat testis homogenates [2,5] and include cytochrome P450 as substrate acceptor and terminal oxidase [6–8]; it has been claimed that both reactions are catalyzed within a single enzyme complex [5].

Testosterone formation from progesterone and other precursors is accepted to occur in the Leydig cells in testis interstitium [2,3,9–12]. There are contradictory results, however, concerning the ability of seminiferous tubules and Sertoli cells to convert progesterone to androgens [13–18].

This study aimed to ascertain the distribution of the 17α-hydroxylase/C-17,20-lyase enzyme activity and of progesterone binding cytochrome P450 within different compartments (seminiferous tubules, interstitial tissue, Leydig cells) prepared from the rat testis. Exact calculation of the impurities within the individual compartments is presented.

2. MATERIALS AND METHODS

Male Wistar rats (200–240 g body wt) were sacrificed by cervical dislocation, testes were decapsulated and incubated in 8 ml of a 1 mg/ml collagenase solution (4 mg/testis; Boehringer, Mannheim) in Eagle's minimum essential medium (MEM) at 33°C for 20 min. The seminiferous tubules and interstitial cell fractions were separated by filtering the collagenase suspensions through 60 μm nylon gauze and subsequent washing in ice-cooled MEM.

In one experimental series, whole decapsulated testes, or the seminiferous tubules or interstitial cell fractions were washed and then homogenized in 0.01 mol/l Tris–HCl, 0.25 mol/l sucrose (pH 7.4) using a Dounce homogenizer. After centrifugation at 10000 × g for 20 min, the supernatant was recentrifuged at 105000 × g for 60 min. The pellet was resuspended in 0.15 mol/l KCl, 0.01 mol/l Tris–HCl (pH 7.4) to obtain the

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microsomal fractions from testes (TM), seminiferous tubules (STM) or interstitial cells (ICM). Protein content in microsomal suspensions was determined using the Lowry method [19] with bovine serum albumin as standard.

In the other experimental series, the interstitial cell suspensions were separated by centrifugation through two-step (1.12 and 1.05 g/ml, respectively) density gradients of Percoll (Pharmacia, Freiburg) at 300 × *g* for 5 min. Both the non-Leydig cell fraction (NLC) and the Leydig cell fraction were washed twice and finally resuspended in MEM or in 9 g NaCl/l. Cell concentrations were determined using a Neubauer chamber. Leydig cells were visualized by staining for 1-naphthyl esterase activity as in [20]. For evaluation of the Leydig cell content in different testicular compartments, either homogenates of the seminiferous tubules (STH) or interstitial cell fractions (ICH), or suspensions of isolated, enriched Leydig cells (LC) or non-Leydig cells (NLC) were processed for quantitative determination of 1-naphthyl esterase activity [20]. In parallel, either tubuli seminiferi (ST) or suspensions of interstitial cells (IC) or purified Leydig cells (LC) were incubated in MEM in presence of 10 mg human chorionic gonadotropin/l (HCG; Primogonyl® , Schering AG, Berlin) for 10 min at 33°C and cyclic AMP formation was measured using a commercially available radioimmunoassay kit (Amersham Buchler, Braunschweig) as in [20].

For measurement of the rates of progesterone consumption by the 17α-hydroxylase/C-17,20-lyase-catalyzed reaction, 10^5 cells (LC or NLC fractions) or 0.1 mg microsomal protein (TM, STM or ICM fractions) were incubated for 5 min at 33°C in presence of different progesterone concentrations (0.3–3.6 μmol/l) and a tracer amount of [^{3}H]progesterone (New England Nuclear, Dreieich; 57 Ci/mmol) in 1 ml total vol. MEM (LC or NLC) or of 1 ml 0.05 mol/l phosphate buffer (pH 7.4) containing 0.3 mmol/l $MgCl_2$ and 0.1 mmol/l NADPH (TM, STM or ICM). Ether extracts were chromatographed on silica gel plates in chloroform/ethylacetate (70:30, v/v) and the progesterone, 17α-progesterone, androstenedione and testosterone fractions were counted for radioactivity. Impurities of the precursor, steroid metabolism in absence of cells or microsomes, and progesterone turnover to metabolites not mentioned above all together amounted to < 4% of the initially added activity. The sum of concentrations of the 3 progesterone metabolites was used as measure for 17α-hydroxylase/C-17,20-lyase activity; V_{max} was derived from double-reciprocal plots of these data.

Progesterone binding to microsomes or isolated cells was measured after induction of type I spectral changes (maximum at 387 nm, minimum at 418 nm) by addition of different amounts (0.2–2 μmol/l) progesterone as the substrate. Difference spectra [6,8] were recorded from 500–360 nm using a Shimadzu UV300 spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto). Maximal spectral differences $A_{387}-A_{418}$ were again derived from double-reciprocal plots of the data and indicated maximal progesterone binding to cytochrome P450.

3. RESULTS AND DISCUSSION

Since we aimed to determine cytochrome P450-dependent hydroxylase activities in different compartments of the rat testis and particularly to prove their presence in the seminiferous tubules, it was necessary either to purify those compartments to homogeneity or at least to quantify possible impurities and to distinguish Leydig cells and non-Leydig cells. For this purpose, both 1-naphthyl esterase activity and HCG-stimulated cyclic AMP formation have been measured (table 1) because both parameters can be used as criteria to identify and quantify rat testis Leydig cells [20]. Both methods give nearly identical results (table 1). The interstitial cell fraction is contaminated by 10.2% Leydig cells, whereas the Leydig cell fraction contains 88.8% and the non-Leydig cell fraction < 0.4% Leydig cells (table 1). It can be further calculated that the seminiferous tubule fraction is contaminated by 1.4% Leydig cells. It is possible to derive from these results the contribution of Leydig cells to progesterone binding cytochrome P450 levels and to progesterone metabolizing enzyme activities in the individual testis compartments (fig.1, hatched parts of the bars).

Rates of progesterone metabolism to 17α-hydroxylated products as well as progesterone binding data give straight lines if plotted against progesterone concentrations in double-reciprocal diagrams. For both the apparent spectra dissocia-

Table 1

Distribution of 1-naphthyl esterase activity and cyclic AMP formation (in presence of 10 mg HCG/ml) within the different fractions prepared from rat testis as in section 2

Compartment	Staining of cells for 1-naphthyl esterase activity (positive cells)	Quantitative determination of 1-naphthyl esterase activity (1-naphthol formation)	HCG-stimulated cyclic AMP formation
ST	–	–	0.2 ± 0.1 pmol.min^{-1}.mg protein^{-1}
STH	–	238 ± 26 nmol.min^{-1}.mg protein^{-1}	–
IC	10.2%	96 ± 11 nmol.min^{-1}.10^6 cells^{-1}	1.5 ± 0.3 pmol.min^{-1}.mg protein^{-1}
ICH	–	558 ± 53 nmol.min^{-1}.mg protein^{-1}	–
NLC	<0.4%	30 ± 3 nmol.min^{-1}.10^6 cells^{-1}	<0.1 pmol.min^{-1}.mg protein^{-1}
LC	88.8%	613 ± 33 nmol.min^{-1}.10^6 cells^{-1}	13.8 ± 2.6 pmol.min^{-1}.mg protein^{-1}
LCH	–	4030 ± 220 nmol.min^{-1}.mg protein^{-1}	–

Values are expressed as means ± SEM of $n = 5$

Abbreviations: ST, seminiferous tubules; STH, homogenates of seminiferous tubules; IC, interstitial cell fraction; ICH, homogenates of interstitial cells; NLC, non-Leydig cell fraction; LC, Leydig cell fraction; LCH, homogenates of Leydig cells

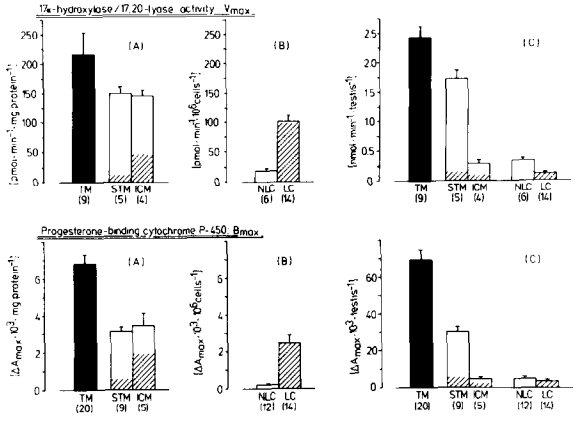

Fig.1. Distribution of 17α-hydroxylase/C-17,20-lyase activity and progesterone-binding cytochrome P450 within rat testis. Data are related either to 1 mg microsomal protein (A) or 10^6 cells (B) of the respective compartments, or to the total of the respective fractions in one testis (C). The hatched parts of the bars indicate the contribution of Leydig cells (calculated from data in table 1) to the respective values. Values are expressed as means ± SEM and numbers of independent experiments are given in parentheses.

Abbreviations: TM, microsomes from whole testis; STM, microsomes from seminiferous tubules; ICM, microsomes from interstitial cells; NLC, non-Leydig cell fraction; LC, Leydig cell fraction.

tion constant (K_s) of progesterone binding to cytochrome P450 and the K_m of the 17α-hydroxylase/C-17,20-lyase system, values of 4 × 10^{-7} mol/l are obtained in good correlation with [6].

Enzyme activities and cytochrome P450 levels as related to 1 mg protein are identical in the microsomal fractions from seminiferous tubules (STM) or from interstitial cells (ICM) (fig.1A). This result is in contrast to reports [18] describing 4-fold higher cytochrome P450 levels and steroid-metabolizing activities in microsomes from interstitial tissue than in microsomes from whole testicular glands; this discrepancy is probably due to the very different methods of interstitial cell preparation.

17α-Hydroxylase/C-17,20-lyase activity in the whole STM compartment of one testis is 5.8-fold higher than in the whole ICM compartment of one testis; for cytochrome P450 content, this relation amounts to 6.7 indicating a nearly parallel distribution of microsomal 17α-hydroxylase/C-17,20-lyase and cytochrome P450 (fig.1C). 87% of the total 17α-hydroxylase/C-17,20-lyase activity and 76% of the total progesterone-binding cytochrome P450 of the rat testis are to be localized within non-Leydig cells (fig.1C, STM and ICM bars).

In the STM and ICM compartments, the ac-

tivities of the enzyme complex (per mg microsomal protein) are only slightly lower, the cytochrome P450 levels are significantly lower ($p < 0.005$) than in microsomes from whole testis (TM) (fig.1A). Preliminary experiments support the hypothesis that this loss of progesterone-binding capacity of cytochrome P450 occurs during the incubation of the testes for collagenase digestion.

Progesterone binding to cytochrome P450 can not only be measured by induction of spectral changes in microsomal suspensions but also in isolated cells (NLC and LC; fig.1B). Although this method has been successfully applied to hepatocytes [21], to our knowledge such determinations have not yet been performed with testicular cells. Both 17α-hydroxylase/C-17,20-lyase activity and progesterone-binding cytochrome P450 concentrations are considerably higher (by 6.1 and 15.1, respectively) in isolated enriched Leydig cells (LC fraction) than in the non-Leydig cell fractions (NLC) of testicular interstitial cells ($p < 0.0005$; fig.1B). The contamination of the NLC fraction by Leydig cells and of the LC fraction by non-Leydig cells can be neglected (table 1, fig.1B). The difference between the relations of LC and NLC levels of cytochrome P450 (= 15.1) and of 17α-hydroxylase/C-17,20-lyase activity (= 6.1) can not conclusively be explained. But it is possible that progesterone induces spectral changes not only at the microsomal, but also at the cytochrome P450 of the mitochondrial cholesterol side-chain cleavage system which is exclusively found in Leydig cells [10]. In adrenocortical mitochondria, type I [22] as well as reverse type I [23] spectral changes are reported after progesterone binding to cytochrome P450.

The observation that both cytochrome P450 content and 17α-hydroxylase/C-17,20-lyase activity are higher in the LC than in the NLC fractions of rat testis interstitium is in accordance with the generally accepted concept that testicular steroidogenesis is mainly associated with the Leydig cells [2,9–12]. Until now, however, a direct comparison of cytochrome P450 content of Leydig cells and non-Leydig cells has not yet been performed. The ability of non-Leydig cells to convert progesterone to 17α-hydroxylated compounds (fig.1) has been observed in seminiferous tubules [13], in Sertoli cells [15,17] and in primary spermatocytes [16] but has not been confirmed by others [14]. Total cytochrome P450 content and total 17α-hydroxylase/C-17,20-lyase activity within the rat testis, however, have now been found to be associated predominantly not with the Leydig cells but with non-Leydig cells of the seminiferous tubules fraction (fig.1C).

The physiological importance of this fact is not clear at present. It has been shown that only Leydig cell testosterone biosynthesis can be stimulated by lutropin [12]. On the other hand, distinct effects of HCG application in vivo on cytochrome P450 content and 17α-hydroxylase/C-17,20-lyase activity have been demonstrated not only in Leydig cells but also in seminiferous tubules from rat testis [24].

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REFERENCES

[1] Slaunwhite, W.R. and Samuels, L.T. (1956) J. Biol. Chem. 220, 341–352.
[2] Tamaoki, B.I. and Shikita, M. (1966) in: Steroid Dynamics (Pincus, G. et al. eds) pp.493–528, Academic Press, London, New York.
[3] Bell, J.B.G., Vinson, G.P., Hopkin, D.J. and Lacy, D. (1968) Biochim. Biophys. Acta 164, 412–420.
[4] Chubb, C. and Ewing, L.L. (1979) Am. J. Physiol. 237, E231–E238.
[5] Samuels, L.T., Bussmann, L., Matsumoto, K. and Huseby, R.A. (1975) J. Ster. Biochem. 6, 291–296.
[6] Menard, R.H. and Purvis, J.L. (1973) Arch. Biochem. Biophys. 154, 8–18.
[7] Betz, G., Tsai, P. and Weakley, R. (1975) Steroids 25, 791–798.
[8] Nakajin, S., Hall, P.F. and Onoda, M. (1981) J. Biol. Chem. 256, 6134–6139.
[9] Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1973) Biochim. Biophys. Acta 297, 179–185.
[10] Wisner, J.R. and Gomes, W.R. (1975) Steroids 26, 769–782.
[11] Van Nimmen, D., Eechaute, W., Lacroix, E., Demeester, G. and Leusen, I. (1979) J. Ster. Biochem. 10, 505–511.

[12] Van der Molen, H.J., Van Beurden, W.M.O., Blankenstein, M.A., De Boer, W., Cooke, B.A., Grootegoed, J.A., Janszen, F.H.A., De Jong, F.H., Mulder, E. and Rommerts, F.F.G. (1979) J. Ster. Biochem. 11, 13–18.
[13] Hall, P.F., Irby, D.C. and DeKretser, D.M. (1969) Endocrinology 84, 488–496.
[14] Dorrington, J.H., Fritz, I.B. and Armstrong, D.T. (1978) Int. J. Androl. Suppl. 2, 53–64.
[15] Steinberger, E., Tcholakian, R.K. and Steinberger, A. (1979) J. Ster. Biochem. 11, 185–192.
[16] Salhanick, A.I. and Terner, C. (1979) Biol. Reprod. 21, 293–300.
[17] Tilbe, K.S. and Wiebe, J.P. (1981) Endocrinology 108, 597–604.
[18] Ohba, H., Inano, H. and Tamaoki, B.I. (1981) Biochem. Biophys. Res. Commun. 103, 1273–1280.
[19] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[20] Kühn-Velten, N., Wolff, J., Passia, D. and Staib, W. (1982) Cell. Mol. Biol. 28, 473–481.
[21] Grundin, R., Moldéus, P., Vadi, H. and Orrenius, S. (1975) in: Cytochromes P450 and b_5. Structure, Function and Interaction (Cooper, D.Y. et al. eds) pp.251–269, Plenum, New York.
[22] Mitani, F. and Horie, S. (1969) J. Biochem. 65, 269–280.
[23] Graves, P.E., Uzgiris, V.I. and Salhanick, H.A. (1980) Steroids 35, 543–559.
[24] Kühn-Velten, N. and Staib, W. (1981) Acta Endocrinol. Suppl. 246, 11–12.