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Differential subcellular distribution of rat prostatic steroid 5α-reductase isozyme activities

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The rat prostate, a classical androgen-target tissue, contains both known isozymes of steroid 5α-reductase, i.e. type I and type II. So far, the role of the type I isozyme has been proposed as catabolic. The abundant expression of type I 5α-reductase in an androgen-target tissue is therefore puzzling. Assessment of the subcellular localization of 5α-reductase isozymes in rat prostate might contribute in elucidating their possibly distinct roles. After obtaining crude subcellular fractions by differential centrifugation, both isozyme activities were measured at neutral pH by plotting according to Eadie–Scatchard. The observations were extended by assessment of pH-dependent velocity ratios and type II 5α-reductase inhibitor sensitivities in these subcellular fractions. The results indicated a preferentially—although not exclusively—nuclear localization for the type I and a predominantly microsomal localization for the type II isozyme activity in the rat prostate. In conclusion, the nuclear localization of the type I isozyme seems not to concur with its proposed catabolic role.

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Steroid 5α-reductase (3-oxo-5α-steroid Δ^4-reductase, E.C. 1.3.1.22) is a membrane-bound, NADPH-dependent enzyme capable of 5α-reducing a number of steroids with a 4,5-double bond and a 3-oxo group, including androgens (1).

The enzyme 5α-reductase was first described in rat liver (2, 3), where it was considered to function as a catabolic enzyme. After 5α-reduction, steroids are susceptible to 3α- and 3β-hydroxysteroid dehydrogenation, sulphation and glucuronylation, facilitating their excretion (1). Later, 5α-reductase activity was also found in rat prostate (4–7), a classical androgen-target tissue. As dihydrotestosterone (DHT) accumulated in the nuclei of prostatic cells after administration of radiolabelled testosterone to rats (4, 8), and as DHT appeared to be a more potent androgen than testosterone in binding to the androgen receptor (9, 10), an anabolic role for the prostatic 5α-reductase has been suggested. To date, two subtypes of steroid 5α-reductase with specific pH optima and inhibitor sensitivities, designated type I and type II, have been described in both human and rat (11–16). Both the catabolic and anabolic roles proposed for 5α-reductase could be attributed to the respected isozymes, based on their tissue-specific localization—type I being found in the liver, type II being the predominant isozyme in the prostate—and based on their respective affinity constants (16).

Studies on the subcellular distribution of 5α-reductase activity in the rat prostate show a wide variety of results. Investigators found nuclear-bound rat prostatic 5α-reductase, yet with a considerable amount of enzyme activity in mitochondrial and microsomal fractions, ranging from 24% to as high as 74% of total activity (6, 17–20). These latter activities, however, might have arisen from nuclear contamination, as has been described for the human prostate (21). Subsequent research focused on the characterization, purification and solubilization of the nuclear-bound prostatic 5α-reductase activity (6, 7, 17, 20, 22). It is now recognized that the rat prostate expresses both isozymes of 5α-reductase (16). Immunocytochemical studies established a nuclear localization of 5α-reductase immunoreactivity—probably attributable to the type I isozyme—in rat prostate epithelium (23). To the best of our knowledge, the subcellular localization of 5α-reductase isozyme activities in rat prostate has not yet been elucidated.

In the present study, the subcellular distribution of 5α-reductase isozyme activities in the rat prostate was investigated to gain better insight into the functional roles of both isozymes. The results suggest a differential subcellular distribution of the two subtypes in this tissue, based on activity measurements, isozyme-specific pH-dependent velocity ratios and inhibitor sensitivities of 5α-reductase activity in crude subcellular fractions.

Materials and methods

Materials

[1,2,6,7-^{3}H]Testosterone (3.74 TBq/mmol) and [1α.2α(*n*)-^{3}H]17β-hydroxy-5α-androstan-3-one (2.00 TBq/mmol) were purchased from Amersham (Amersham, UK). [9,11-^{3}H]5α-Androstane-3α,17β-diol (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high-performance liquid chromatography (HPLC, see below) before use. Testosterone was purchased from Steraloids (Wilton, NH). Diethylether (p.a.), *n*-hexane (LiChrosolv) and 2-propanol (LiChrosolv) were purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade. The specific rat 5α-reductase type II inhibitor L-685,273 (21,21-pentamethylene-4-aza-5α-pregn-1-ene-3,20-dione) was a kind gift from Merck, Sharp and Dohme Corp. (Rahway, NJ).

Buffers

The homogenization buffer consisted of 20 mmol/l phosphate (Merck), 1 mmol/l monothioglycerol, 50 μmol/l NADPH tetrasodium salt (Merck) and 0.25 mol/l sucrose (Merck) (pH 7.0). The incubation buffer consisted of 200 mmol/l TRIS (2-amino-2-hydroxymethylpropane-1,3-diol, Merck)/citric acid monohydrate (Merck) and 2 mmol/l NADPH (pH 5.0 or 7.0).

Animals and tissue preparation

Ten Wistar rats from the local breeding facility of 7–13 weeks old (150–250 g) were killed by decapitation and whole prostates were removed, freed of adhering fat and placed in liquid nitrogen for transport to our laboratory. Tissues were processed on the same day. All subsequent procedures were performed on ice. Pooled tissues (2.3 g total wet wt) were minced with razor blades and homogenized in ice-cold homogenization buffer with a 7-ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ) with a loose- and a tight-fitting pestle. The homogenate was filtered twice through nylon netting of 50 and 140 mesh, respectively, to remove cell debris. This full homogenate was diluted with homogenization buffer to a concentration of 21 mg protein/ml.

Preparation of subcellular fractions

Crude subcellular fractions were obtained by differential centrifugation at 4°C. After centrifugation at 1000 *g* for 15 min in a Sorvall RC24 with a SM24 fixed-angle rotor, a crude nuclear pellet was obtained. The resulting supernatant was centrifuged at 10 000 *g* for 20 min in the same rotor to obtain a mitochondrial pellet. The 10 000 *g* supernatant was centrifuged subsequently at 108 000 *g* for 60 min in an MSE Prepspin 75 with a SP-2818 fixed-angle rotor, resulting in a microsomal pellet and a supernatant containing the cytosol. All pellets were resuspended in homogenization buffer with a glass–glass tissue grinder (Kontes) to 4 (mitochondrial fraction), 8 (microsomal fraction) and 9 mg protein/ml (crude nuclear fraction). Aliquots of 1 ml were stored at −80°C and assayed within 4 weeks.

Protein levels were determined by a method modified from Lowry et al. (24) against a standard of bovine serum albumin (OHRD 20/21, Hoechst-Behring, Marburg, Germany). The assay was modified for microtitre plates and had a sensitivity of 25 μg per well.

5α-Reductase assay

The assay has been described and validated in detail in earlier papers (25, 26). In short, radiolabelled testosterone was diluted isotopically to a range of 2 nmol/l–3 μmol/l (final concentration, 10 duplicate points), dissolved in incubation buffer (800 μl) in Pyrex culture tubes (borosilicate glass, 12 × 75 mm, Corning Inc., NY) and subsequently preheated at 37°C. For the inhibition experiments, the specific rat 5α-reductase type II inhibitor L-685,273 was added from stock solutions in ethanol (10 pmol/l–10 μmol/l final concentration) simultaneously with testosterone (30 nmol/l final concentration). This inhibitor is type II specific with inhibition constants of 0.04 nmol/l for type II and 2.8 nmol/l for type 1 5α-reductase, respectively (16). Ten to 100 μl of the enzyme preparation (40 μg–2.1 mg protein) and the appropriate amount of co-factor (NADPH, 2 nmol/l final concentration) was supplemented to 200 μl with incubation buffer and added to the tubes with substrate to start the incubation. After 30 min the incubation was terminated by adding 100 μl of 3 mol/l NaOH, and metabolites were extracted with 4 ml of ice-cold diethyl ether. Percentile metabolism ranged from 1% to 16%. Extracted metabolites were reconstituted in 100 μl of hexane for HPLC.

High-performance liquid chromatography

Metabolized steroids were separated on a Hibar LiChrosorb Diol column (length 250 mm, 5 μm, Merck) equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC system included a Waters 610 fluid unit, a Waters 600E system controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane/propanol 96 : 4, v/v) was 1.5 ml/min at a pressure of 680 psi. Radioactivity was monitored with a FloOne Beta Radiomatic A500 radio-chromatography detector (Packard-Canberra Benelux, Tilburg, The Netherlands) with a 500-μl cell and a liquid scintillation flow of 1.5 ml/min (Aqua-Luma, Lumac-LSC, Olen, Belgium). The counting efficiency for tritium was 47%. The percentage formation

of DHT and 5α-androstane-3α(β)17β-diol (Adiol) was used to estimate 5α-reductase activity.

Calculation of enzyme characteristics

Velocities were plotted against testosterone concentrations (over 10 duplicate points), and K_m and V_{max} values were calculated using a non-linear regression procedure to least-squares, based on a Michaelis–Menten equation for two independent isozyme activities, with the computer program Enzfitter. A double reciprocal plot (Lineweaver–Burk) of the estimated initial velocities obtained against substrate concentration was used. Furthermore, an Eadie–Scatchard plot of velocity over substrate concentration against velocity was also used, as this plot is reportedly best suited to detect isozyme activities (27). The IC_{50} values were determined from plots of enzyme velocity against inhibitor concentration.

Statistical analysis

Values were obtained in at least two duplicate assays carried out on different days. Statistical analysis was performed using a Pearson correlation test or analysis of variance and, when ANOVA revealed a statistically significant difference, a subsequent two-tailed Bonferroni (Dunn) *t*-testing with the SAS statistical software program.

Results

The validity of the 5α-reductase assay was established and reported in earlier papers (25, 26). Initial velocity data were obtained at a substrate concentration range of 2 nmol/l–3 μmol/l testosterone (Fig. 1A) and plotted according to Lineweaver–Burk (Fig. 1B) and to Eadie–Scatchard (Fig. 1C). The best fits for isozyme activities are presented as solid lines in these figures. Lineweaver–Burk plots deviated from linearity only at the higher substrate concentrations. Eadie–Scatchard plots, however, were clearly non-linear and could be described by two enzyme activities as shown by two additional lines in Fig. 1C. All subcellular fractions exhibited similar non-linear plots (data not shown).

In Table 1 the data obtained in crude subcellular fractions from rat prostate are summarized. Approximately 70% of total protein was recovered in the subcellular fractions. The total recovery of enzymatic activity was 72.4% for type I and 73.7% for type II 5α-reductase, respectively. For the type I isozyme, analysis of variance revealed differences in yield between fractions ($p < 0.002$). The yield of type I activity was highest in the nuclear fraction (Fig. 2A). The mitochondrial and microsomal fraction contained three-to fourfold less type I 5α-reductase activity as compared to the nuclear fraction ($p < 0.005$ by two-sided Bonferroni *t*-test). No significant differences in yield were found for the type II isozyme between the subcellular fractions ($p > 0.4$ by ANOVA), approximately 25% of total activity being present in each of the three fractions. No 5α-reductase activity was found in the 108 000 *g* supernatant (cytosol).

For both isozymes the specific activities in the subcellular fractions were all higher than in the full homogenate (Table 1). The enrichment values for the isozymes, calculated as the ratio of specific activity in the subcellular fraction over that in the full

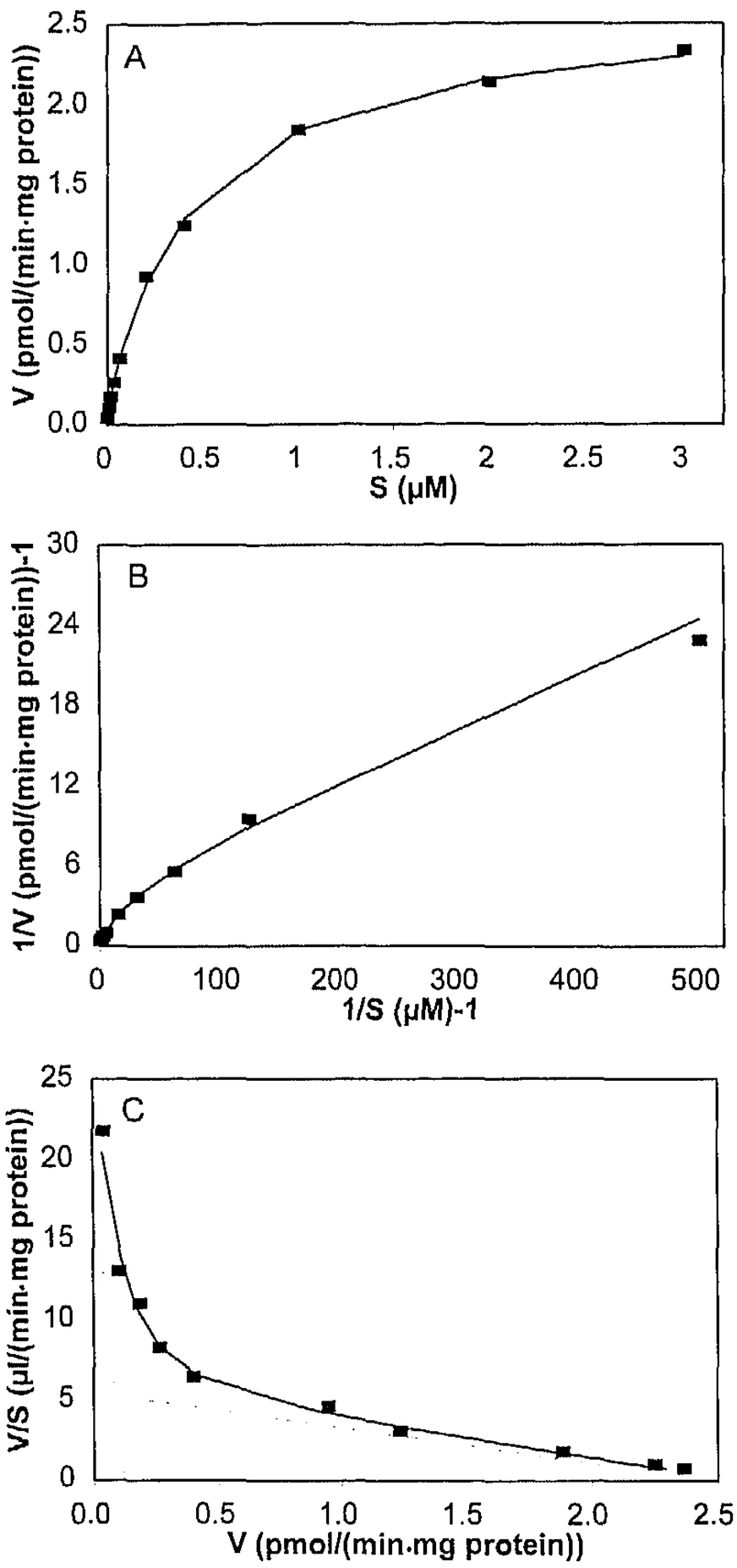

Fig. 1. Detecting isozyme activities of rat prostatic 5α-reductase. Best fit for isozyme activity is presented by solid lines. (A) Initial velocities (V) were obtained at 2 nmol/l–3 μmol/l testosterone and 2 mmol/l NADPH at 37°C in a full homogenate of rat prostate. Values are means of at least two duplicate experiments carried out on two different days. (B) A double-reciprocal (Lineweaver–Burk) plot of these data deviates from linearity only at high testosterone concentrations. (C) An Eadie–Scatchard plot of V/substrate concentration (S) against V is clearly non-linear. Activities of type I (– – –) and type II (- - -) 5α-reductase isozymes can be calculated from these data.

Table 1. Protein and 5α-reductase isozyme data obtained in crude subcellular fractions of rat prostate.

Subtype	Protein (mg) (%)	Activity (pmol/min) (%)		Specific activity (pmol · min^{-1} · mg^{-1} protein)		K_m (nmol/l)	
		I	II	I	II	I	II
Full homogenate	341.7 (100)	1010.7 (100)	48.2 (100)	2.96	0.141	528	7.2
Nuclei (1000 *g* pellet)	59.2 (16.5)	472.2 (46.7)	13.4 (27.8)	7.97	0.226	613	4.9
Mitochondria (10 000 *g* pellet)	20.1 (5.8)	150.7 (14.9)	10.8 (22.4)	7.50	0.538	458	4.8
Microsomes (108 000 *g* pellet)	15.7 (4.6)	109.4 (10.8)	11.3 (23.5)	6.97	0.722	577	5.2
Cytosol (108 000 *g* supernatant)	149.7 (43.0)	–	–	–	–		

homogenate, are shown in Fig. 2B. No significant differences between the values in nuclei, mitochondria and microsomes can be seen for the type I isozyme ($p > 0.7$ by ANOVA). The enrichment for this isozyme was approximately 2.5 in all three fractions. For the type II isozyme activity the enrichment differed between fractions ($p < 0.02$ by ANOVA). Highest purification was achieved in the microsomal fraction (five times higher specific activity), and lowest enrichment (1.6 times) in the nuclear fraction. The values for the type II isozyme in the mitochondrial and microsomal fractions were significantly higher than that in the nuclear fraction ($p < 0.1$ and $p < 0.05$, respectively, by the two-sided Bonferroni *t*-test).

The affinity constants for the type I isozyme were approximately 500–600 nmol/l in the full homogenate as well as in the subcellular fractions (Table 1). The type II isozyme activity had a K_m of approximately 5 nmol/l. These affinity constants did not differ significantly between the subcellular fractions ($p > 0.8$ by ANOVA).

To corroborate the isozyme activity assay and to substantiate further the subcellular distribution results, two other characteristics of 5α-reductase isozymes, i.e. pH dependency of velocities and inhibitor sensitivity, were investigated in the same subcellular fractions. The ratios of V_{max} values of both isozyme activities obtained with Eadie–Scatchard plots (V_{max} I/II) in the subcellular fractions were correlated with isozyme-specific pH-dependent velocity ratios (v pH 7.0/pH 5.0) (Fig. 3A). The activities at pH 7.0 and pH 5.0 are generally considered indicative of type I and type II isozyme activity, respectively (28). Initial velocities were estimated with 1 μmol/l testosterone and 2 nmol/l NADPH at pH 7.0 and at pH 5.0. A high positive correlation of 0.942 ($p < 0.025$) between the ratios in the different subcellular fractions was found using the Pearson correlation test. The microsomal fraction exhibited the lowest isozyme type I over type II V_{max} ratio and isozyme-specific pH-dependent velocity ratio (approximately 10 and 0.32, respectively); the crude nuclear fraction, on the other hand, exhibited the highest values (approximately 35 and 0.78, respectively). The

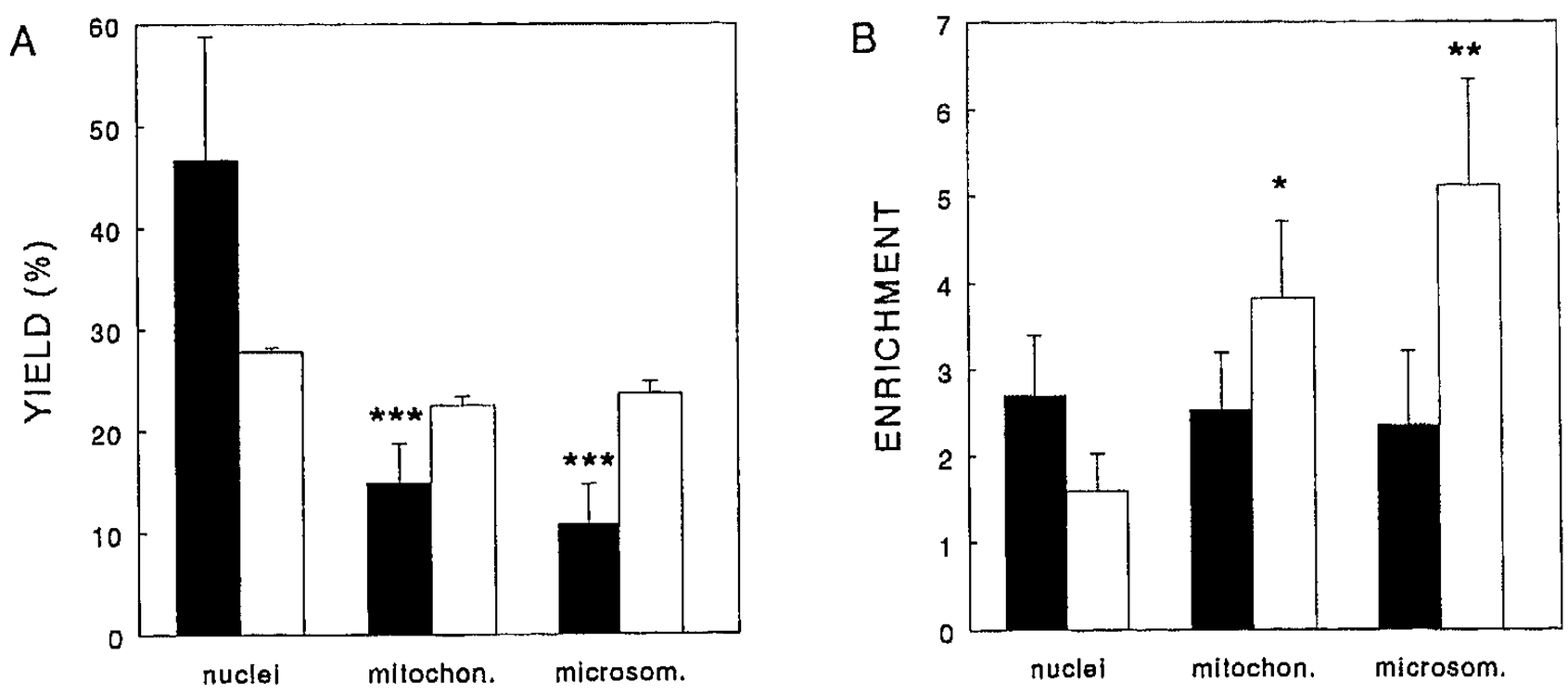

Fig. 2. 5α-Reductase activities in crude subcellular fractions of rat prostate. Values are presented for the type I (solid bars) and type II (open bars) isozyme activities. (A) Percentile 5α-reductase isozyme activity (yield) in the different subcellular fractions. Values are means + SD of at least two duplicate assays carried out on different days. The recoveries of type I activity in mitochondrial and microsomal fractions were significantly less as compared to the nuclear fraction. No significant differences between fractions were found for the type II isozyme activities. (B) Enrichment values (relative specific activities) were calculated as the ratio of specific activity in the subcellular fractions over that in the full homogenate. Values are means + SD of at least two duplicate assays carried out on different days. The type II isozyme showed significantly higher purification in the mitochondrial and microsomal fractions as compared to the nuclear fraction. No significant differences between fractions were found for the type I isozyme activities; *$p < 0.1$, **$p < 0.05$ and ***$p < 0.005$.

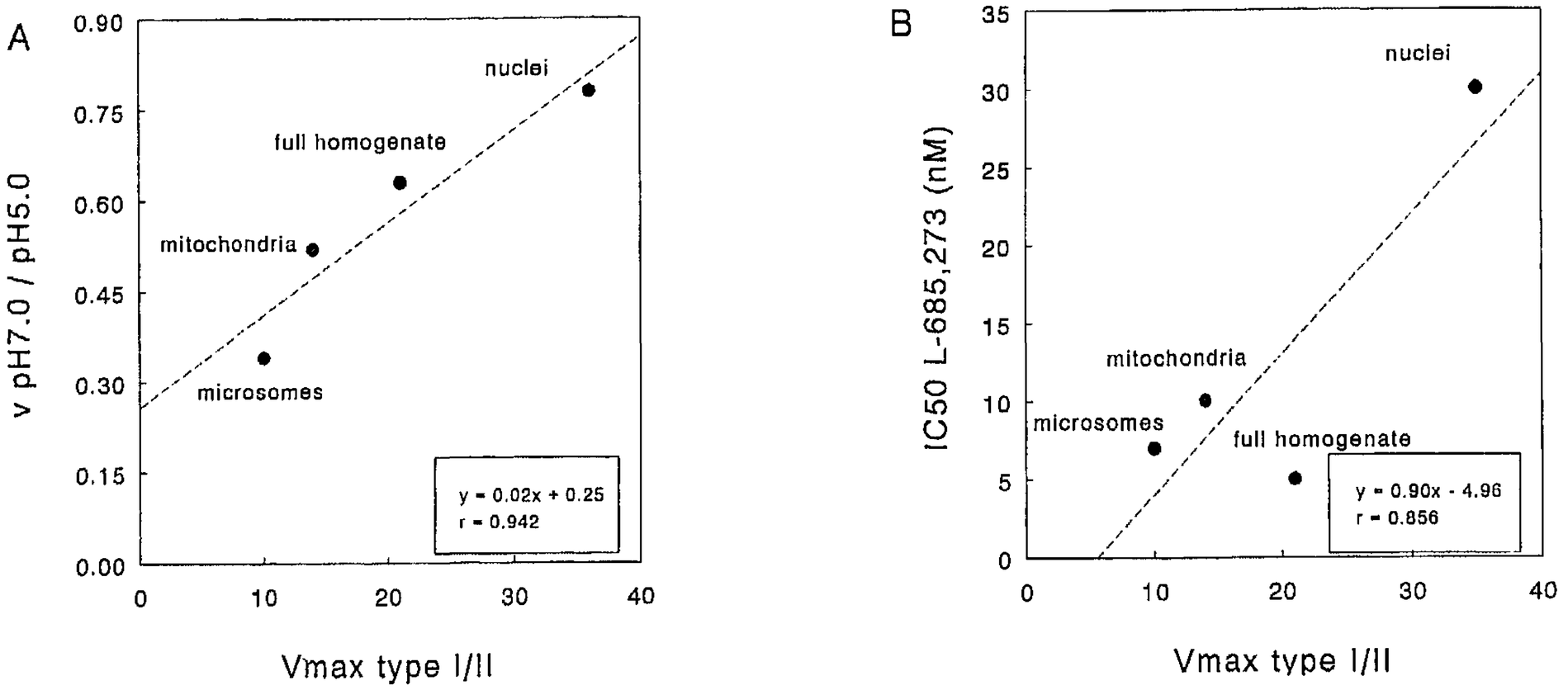

Fig. 3. Validation of isozyme activity measurements and their subcellular distribution. (A) Initial velocities were obtained with 1 μmol/l testosterone and 2 mmol/l NADPH at both pH 7.0 (≈type I) and pH 5.0 (≈type II) in subcellular fractions of rat prostate. The ratio of velocities at pH 7.0 over pH 5.0 (v pH 7/pH 5.0) were correlated with isozyme V_{max} ratio values calculated as described in Fig. 1 (V_{max} I/II). A positive correlation of 0.942 ($p < 0.025$) was found using the Pearson correlation test. (B) Full homogenate, crude nuclear, mitochondrial and microsomal fractions were incubated as described in the Materials and Methods section with 30 nmol/l testosterone and 10 pmol/l–10 μmol/l of the specific rat 5α-reductase type II inhibitor L-685,273. The IC_{50} values were calculated and correlated with isozyme V_{max} ratio values obtained as described in Fig. 1. A positive correlation of 0.856 ($p < 0.05$) was found using the Pearson correlation test.

10 000 *g* pellet (mitochondria) and the full homogenate exhibited intermediate values.

Because of the differences in affinity constants for testosterone, the contribution to total 5α-reductase activity of each isozyme depends on the substrate concentration. The testerone concentration used for the inhibition experiments (30 nmol/l was chosen so that each isozyme would have an almost similar contribution to total 5α-reductase activity. This relative contribution will depend on the ratio of isozyme activities. Therefore, differences in IC_{50} values at a fixed substrate concentration can be attributed to differences in isozyme activity ratios in the subcellular fractions. Initial velocities were estimated at 10 pmol/l–10 μmmol/l of the specific rat 5α-reductase type II inhibitor L-685,273. The IC_{50} values were 5, 10 and 7 nmol/l for the full homogenate, mitochondrial and microsomal fractions, respectively, but 30 nmol/l for the crude nuclear fraction. As shown in Fig. 3B, the correlation between these IC_{50} values and the type I over type II V_{max} ratios was statistically significant (0.856; $p < 0.05$, Pearson correlation test).

Discussion

To gain better insight into the differential roles of the two hitherto known 5α-reductase isozymes expressed in the rat prostate (16), the activities of both isozymes were evaluated in crude subcellular fractions. The rat prostate has been used as a model for the human prostate because growth and development of both tissues are androgen-dependent, both contain high 5α-reductase activity and because of the notable similarity between the human and rat 5α-reductase subtypes.

The rat and human type I 5α-reductase isozymes have been reported to have a K_m for testosterone of about 0.5–1 μmol/l (16, 25, 26), whereas the type II isozymes have a K_m, at neutral pH, of 4–50 nmol/l (25, 26, 29, 30). As the substrate concentration should ideally be in the neighbourhood of the K_m (27), subcellular fractions of the rat prostate were incubated with a wide range of testosterone concentrations of 2 nmol/l–3 μmol/l. A Lineweaver–Burk plot of these data only deviated from linearity at high substrate concentrations, whereas Eadie-Scatchard plots were clearly non-linear over essentially the whole substrate range tested.

The non-linearity of these plots might indicate negative cooperative binding of testosterone to 5α-reductase (27). We propose, however, that this non-linearity can be explained better by the presence of two 5α-reductase isozyme activities. The affinity constants obtained (approximately 500 and 5 nmol/l for the alleged type I and type II activities, respectively) are similar to those reported in the literature for the rat and human steroid 5α-reductase isozymes at neutral pH (16, 25, 26, 29, 30). In addition, data obtained with Eadie–Scatchard plots and isozyme-specific pH-dependent enzyme velocity ratios were closely related. Generally, the type II isozyme is considered to have an acidic pH optimum (16). Velocities at pH 5.0 do not properly reflect type II in vivo activity, as this isozyme is now considered to operate at neutral pH (30). The ratio between activity at pH 7.0 and pH 5.0, however, does represent differences in isozyme activity ratios (28). The

correlation between data obtained by Eadie–Scatchard plots at pH 7.0 and the ratios of enzyme activity at pH 7.0 and pH 5.0 was indeed highly significant. Furthermore, the correlation between isozyme V_{max} ratios and IC_{50} values of the type II-specific inhibitor L-685,273 in the subcellular fractions of the rat prostate was also significant. Altogether, these data strongly indicate the presence of two isozyme activities, resulting in the non-linear Eadie–Scatchard plots.

The sub-cellular localization of 5α-reductase in the rat prostate has been considered nuclear, both for enzymatic activity (6, 7, 17, 20, 22) and immunoreactivity (23). In the rat liver, type I 5α-reductase has been localized in the microsomal fraction (17), although this has been contended in a recent paper (31). To the best of our knowledge, the subcellular localization of the isozyme activities in the rat prostate has not yet been elucidated. The results presented in this paper indicate a differential subcellular distribution of the two known subtypes of 5α-reductase in the rat prostate. Highest purification was obtained for the type II isozyme in the microsomal fraction. Furthermore, almost half of the type I activity sedimented in the 1000 *g* pellet. However, the specific activity of the type I isozyme was similar in all three subfractions. This might be attributed to the difficulty in obtaining pure subcellular fractions due to the structural continuity between the outer nuclear membrane and the rough endoplasmatic reticulum, which has also led to controversy on the subcellular localization of the 5α-reductase type I isozyme in rat liver (31). The 5α-reductase enzymatic activity found in the 10 000 *g* pellet (crude mitochondrial fraction) can be attributed to contamination with nuclear and microsomal membrane particles. This "mitochondrial" 5α-reductase has also been found by other authors applying differential centrifugation to obtain subcellular fractions (6, 18, 20). Although the present study does not overcome the contamination problems associated with differential centrifugation as reported for the human prostate (21), either isozyme activity can serve as a marker for the other. If the isozyme activity ratio differs between subcellular fractions, as indeed is described here, a differential subcellular distribution can be surmised. This ratio of isozyme activity can only differ between fractions if the isozymes are differentially localized. Furthermore, the results on pH-dependent velocity ratios and inhibitor sensitivities in the crude subcellular fractions confirmed our conclusion on the subcellular distribution of isozyme activities. Surprisingly, it was the type I isozyme that appeared predominantly—although not exclusively—nuclear-bound, whereas the type II isozyme had a more microsomal localization.

Dihydrotestosterone formed by the nuclear type I isozyme will more likely bind to the androgen receptor, also located in the nucleus (32, 33), than that formed by the microsomal type II isozyme. This suggests an anabolic role for the 5α-reductase type I in this tissue. Because in the rat prostate a high amount of 3α/β-hydroxysteroid dehydrogenase (HSOR) activity is present in the cytoplasm (34), any DHT formed by the type II in the endoplasmatic reticulum would instantly be degraded to Adiol. Therefore, our results unexpectedly suggested a possibly catabolic role for the type II isozyme in the rat prostate.

However, in hypothesizing about the function of the 5α-reductase isozymes, their cell-type specific localization should also be considered. Type I 5α-reductase is expressed in the epithelial cells of the rat prostate (23, 35). In the DHT-dependent epithelium (36), nuclear-bound 5α-reductase type I (this study) would metabolize testosterone to DHT to bind to the androgen receptor, suggesting a role of this subtype in the intracrinology of this tissue (42). On the other hand, DHT formed by the type II in the endoplasmatic reticulum (this study) of rat prostatic stromal cells (35) is metabolized extensively to Adiol (34), which has been shown to induce benign prostatic hyperplasia (BPH) in the dog (39). Stromal signals act on the epithelium in the development of certain glands (37, 38). Dihydrotestosterone and Adiol formed by the type II isozyme could therefore act as paracrine factors in this tissue, influencing epithelial cell fate.

Finally, another possible distinction in the roles of 5α-reductase might be considered. The high affinity of the type II 5α-reductase isozyme makes it well suited for metabolizing the low concentrations of testosterone in fetal life (30), leading to the hypothesis that it is the type II isozyme that is responsible for the development of the prostate. At the higher testosterone concentrations in adulthood, type I 5α-reductase might also contribute to DHT formation, and therefore be responsible for growth and maintenance of the prostate (35). Because of the poor development of prostates in patients with 5α-reductase type II deficiency (pseudohermaphroditism) (40), a type II inhibitor (Finasteride, Proscar®) has been developed for treatment of patients with BPH symptoms (41). Recently, in addition to type II, we reported type I 5α-reductase activity in human prostate with BPH (26), necessitating research into this subtype and into its possible involvement in the pathogenesis of BPH.

In conclusion, Eadie–Scatchard plots of 5α-reductase activities, pH-dependent isozyme-specific velocity ratios and inhibitor sensitivities reported in this paper indicate a differential distribution of isozyme activities, i.e. a nuclear localization of the type I and a microsomal localization of the type II 5α-reductase isozyme in the rat prostate. To elucidate their roles, the cell-type specific localization of isozyme activities and the expression during ontogeny should also be taken into account.

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