New Evidence of Similarity between Human and Plant Steroid Metabolism: 5alpha-Reductase Activity in Solanum malacoxylon

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New Evidence of Similarity between Human and Plant Steroid Metabolism: 5α-Reductase Activity in *Solanum malacoxylon*

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The physiological role of steroid hormones in humans is well known, and the metabolic pathway and mechanisms of action are almost completely elucidated. The role of plant steroid hormones, brassinosteroids, is less known, but an increasing amount of data on brassinosteroid biosynthesis is showing unexpected similarities between human and plant steroid metabolic pathways. Here we focus our attention on the enzyme 5α-reductase (5αR) for which a plant ortholog of the mammalian system, DET2, was recently described in *Arabidopsis thaliana*. We demonstrate that campestenone, the natural substrate of DET2, is reduced to 5α-campestanone by both human 5αR isozymes but with different affinities. *Solanum malacoxylon*, which is a calcinogenic plant very active in the biosynthesis of vitamin D-like molecules and sterols, was used to study 5αR activity. Leaves and calli were chosen as examples of differentiated and undifferentiated tissues, respectively. Two separate 5αR activities were found in calli and leaves of *Solanum* using campestenone as substrate. The use of progesterone allowed the detection of both activities in calli. Support for the existence of two 5αR isozymes in *S. malacoxylon* was provided by the differential actions of inhibitors of the human 5αR in calli and leaves. The evidence for the presence of two isozymes in different plant tissues extends the analogies between plant and mammalian steroid metabolic pathways. (*Endocrinology* 144: 220–229, 2003)

THE HUMAN 5α-REDUCTASE (5αR) is a system of two membrane-bound isozymes that catalyzes the selective and irreversible reduction of 4-ene-3-oxosteroids to the corresponding 5α-3-oxosteroids. 5αR is primarily involved in androgen synthesis, being responsible for the conversion of testosterone into 5α-dihydrotestosterone, the androgen with the highest affinity for the androgen receptor. Two human isozymes of 5αR have been cloned, expressed, and characterized: They have different chromosomal localization, enzyme kinetic parameters, pH optima, and tissue expression patterns (1).

The type 2 isozyme (5αR-2) has nanomolar affinity for testosterone, and it is found predominantly in prostate, genital skin, seminal vesicles, and epididymis. Moreover, it is present in the outer root sheet of the hair follicle and dermal papilla, and it is transiently expressed in the brain during the perinatal period (2–6). 5αR-2 is essential for differentiation of male external genitalia during fetal life, as assessed by studies on male pseudohermaphroditism in which total or partial deficiency of 5αR-2 has been found (7).

The type 1 isozyme (5αR-1) has micromolar affinity for testosterone; occurs predominantly in the sebaceous gland, liver, and brain; and is present in the hair follicle and prostate (8, 9). The two 5αR isozymes are differently involved in prostatic diseases (benign prostatic hyperplasia and prostate cancer) and skin-related diseases (acne, hirsutism, and androgenetic alopecia) (10). The inhibition of 5αR isozymes has represented an attractive pharmaceutical target during the last 20 years, and many classes of steroidal and nonsteroidal 5αR inhibitors have been described with different degrees of selectivity toward the two isozymes (11–15). The homology between the two 5αR isozymes is in fact poor (50% ca.), and it is possible to find molecules that selectively inhibit only one of the two isozymes, demonstrating that sequence differences are located in the protein binding domain.

In addition to testosterone, the 5αR system is able to reduce other 4-ene-3-oxosteroids, *e.g.* progesterone. This steroid is preferentially reduced in the brain with the formation of dihydroprogesterone that binds the nuclear progesterone receptor with high affinity. Dihydroprogesterone is successively reduced by 3α-hydroxysteroid dehydrogenase to tetrahydroprogesterone, which is the most potent natural neurosteroid and a potentiator of γ-aminobutyric $acid_A$ receptor activation (16, 17).

The 5αR enzyme system is present in many other mammalian species including monkeys, dogs, rats, and mice (18–22). In all these species, the catalyzed reaction is the same as in humans with the main product being 5α-dihydrotestosterone, and this is an indication of a common function of androgen activation. The comparison between the human isozymes and 5αRs of other species shows a shared homology ranging from 70% (rat) to 90% (monkey). The kinetic

Abbreviations: Campestenone, 24(R)-24-methylcholest-4-en-3-one; campestanone, 24(R)-24-methyl-5α-cholestan-3-one; CHO, Chinese hamster ovary; DTT, dithiothreitol; 4-MA, 17β-(*N*,*N*-diethyl)carbamoyl-4-methyl-4-aza-5α-androstan-3-one; NADPH, β-nicotinamide adenine dinucleotide 3′-phosphate reduced form; NMR, nuclear magnetic resonance; NOG, n-octyl-β-D-glucopyranoside; PMSF, phenylmethylsulfonyl fluoride; 5αR, 5α-reductase; 5αR-1, 5αR isozyme 1; 5αR-2, 5αR isozyme 2; TLC, thin layer chromatography.

properties of the two 5αR isozymes, and in particular the response to inhibitors, are species specific. In all these species, there is a specific tissue distribution pattern of the two 5αR isozymes, and the fact that all these mammals possess two 5αR genes indicates that the duplication event that gave rise to the type 1 and 2 enzymes occurred early in evolution (23).

Recently 5αR activity has been found also in plants: Li *et al.* (24) have isolated a gene in *Arabidopsis thaliana*, named *DET2*, that encodes a protein sharing about 40% sequence identity with both human 5αRs. The sequence similarity increases to 60% when conservative substitutions are taken into account, and 80% of the absolutely conserved residues in mammalian enzymes are found in the predicted DET2 protein. *Arabidopsis* mutants having a loss-of-function mutation in the *DET2* gene are named *det2* and are small, dark green dwarf plants displaying pleiotropic defects in light-regulated development: they have the characteristics of light-grown plants even when grown in the dark. The mutant phenotype can be reverted by application of brassinolide, the most active molecule belonging to brassinosteroids, a ubiquitous class of plant steroid hormones (25). All known biologically active brassinosteroids possess a 5α-reduced stereochemistry, and it has been demonstrated that DET2 catalyzes the reduction of 24(R)-24-methylcholest-4-en-3-one (campestenone) to 24(R)-24-methyl-5α-cholestan-3-one (5α-campestanone) in the biosynthetic pathway of brassinolide. Thus, in analogy with mammals 5αRs, DET2 is involved in the biosynthesis of plant steroid hormones (26). In addition, the study of *det2* mutants revealed that two of the mutants have a nonconservative substitution of lysine for glutamate at position 204, *i.e.* corresponding to the missense mutation that causes a conservative substitution of aspartate for glutamate 197 identified in the human 5αR-2 gene from families affected by 5αR-2 deficiency (24).

The expression of recombinant DET2 protein in human embryonic kidney 293 cells allowed the kinetic characterization of the enzyme, and it has been demonstrated that DET2 is able to reduce typical substrates of mammalian 5αRs like testosterone and progesterone. On the other hand, the cDNA encoding either type 1 or type 2 human 5αRs, when stably introduced in *det2-1* mutants using *Agrobacterium*-mediated transformation, was able to complement the *det2* mutation in plants and rescue the mutant phenotypes (27).

All these structural and functional similarities indicate that DET2 is an ortholog of the mammalian 5αRs and that probably all these enzymes evolved from a common ancestor (28). A conserved pathway for the synthesis of steroid hormones suggests that the function of these molecules in signal transduction evolved before plants and animals diverged from protists.

In the present study, we focused our attention to the 5αR system in plants defining its similarities with the human enzymes. *Solanum malacoxylon*, which is a calcinogenic plant very active in the biosynthesis of vitamin D-like molecules and sterols, was chosen for this study. We used the natural DET2 substrate, campestenone, to study the kinetic characteristics of plant 5αR and directly demonstrate that the plant substrate is reduced by the human isozymes. Substrates and inhibitors of human 5αRs were also used to characterize the plant enzyme in a better way.

Materials and Methods

Materials

CHO (Chinese hamster ovary) cell lines CHO1827 and CHO1829, stably transfected with human 5αR-1 and 5αR-2, respectively, were obtained from Serono International.

The radioactive substrates [7-^{3}H(N)] androst-4-ene-3,17-dione (24 Ci/mmol), [1,2,6,7-^{3}H(N)] progesterone (97 Ci/mmol), [1,2,6,7-^{3}H(N)] testosterone (80 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). Unlabeled steroids (progesterone, testosterone, androstenedione), campesterol, β-nicotinamide adenine dinucleotide 3′-phosphate reduced form (NADPH), BSA fraction V, Bradford reagent, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and n-octyl-β-D-glucopyranoside (NOG), were purchased from Sigma-Aldrich (St. Louis, MO). Instagel plus was from Packard (Meridien, CT).

Finasteride (17β-(*N*,t-butyl)carbamoyl-4-aza-5α-androst-1-en-3-one) was a kind gift of Merck Sharp & Dohme Research Laboratories. 8-Chloro-4-methyl-1,2,3,4,4a,5,6,10b-octaahydro-benzo[f]quinolin-3(2H)-one) and 4-MA (17β-(*N,N*-diethyl)carbamoyl-4-methyl-4-aza-5α-androstan-3-one) were synthesized in our laboratories according to the described methods (29, 30). The 10-azasteroid [(+)-17-(3-Pyridyl)-(5β)-10-azaestra-1,16-dien-3-one] and the nonsteroid inhibitor 8-chloro-benzo[c]quinoliz-3-one were synthesized as previously reported by us (15, 31).

24(R)-24-methylcholest-4-en-3-one (campestenone) and 24(R)-24-methyl-5α-cholestan-3-one (5α-campestanone) were prepared according to the described methods (32, 33), and the syntheses are briefly described in *Methods.* (24*R*)-24-methyl-[2,4,6(n)-^{3}H]cholest-4-en-3-one (67 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

^{1}H nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ at 200 MHz, on a Varian Gemini (Fort Collins, CO) instrument. HPLC analyses were performed on a Beckman-Gold system (Beckman, Fullerton, CA) equipped with analytical Econosphere C18 10 μm, 250 × 4.6 mm, reverse-phase column (Alltech, Deerfield, IL) using an H_2O-CH_3CN gradient eluant. Signals were monitored at 254 nm with a UV detector. Thin-layer chromatography (TLC) was performed on TLC plates (Merck, Darmstadt, Germany) (20 × 20 cm) silica gel 60 F254. Radioactive samples were counted in a liquid scintillation counter Betamatic V (Kontron Instruments Ltd., Watford, UK).

Methods

*Synthesis of (24R)-24-methylcholest-4-en-3-one and (24*R*)-24-methyl-5α-cholestan-3-one.*

*(24*R*)-24-methylcholest-4-en-3-one.* Toluene (2 ml) was distilled under a nitrogen atmosphere from a refluxing solution of campesterol (25 mg, 0.062 mmol) and 1-methyl-4-piperidone (42.5 μl) in toluene (3 ml). Aluminum isopropoxide (13.5 mg) was then added in one portion. The reaction mixture was refluxed for 4 h; cooled; diluted with ether; and successively washed with 10% aqueous citric acid, water, saturated aqueous $KHCO_3$, and water. After solvent evaporation, the product (24.4 mg, 98% yield) was obtained pure as indicated by analytical HPLC (flow = 1 ml/min, 30–95% CH_3CN/10 min, t_R = 44.2 min); ^{1}H NMR ($CDCl_3$) δ 5.69 (singlet, 1 H), 2.40–0.68 (m, 45 H); analytically calculated for $C_{28}H_{46}O$: C, 84.36; H, 11.63; found: C, 84.38; H, 11.79.

(24R)-24-methyl-5α-cholestan-3-one. A suspension of lithium (12 mg) in ethylamine (1 ml) was stirred at −78 C for 30 min. A solution of (24*R*)-24-methyl-cholest-4-en-3-one (10 mg, 0.025 mmol) and *t*-butanol (40 μl) in dry tetrahydrofuran (1 ml) was added dropwise, then the reaction mixture was stirred for additional 30 min at −78 C. Water (500 μl) was added, and the mixture was allowed to warm at room temperature. Additional water was added and the mixture extracted with ether. Purification by preparative TLC (eluant CH_2Cl_2-Et_2O 32:1, R_f = 0.75) afforded the final product as a yellowish solid (3.3 mg, 33%); ^{1}H NMR ($CDCl_3$) δ 2.38–0.65 (m, 48 H); MS *m/z* 400 (M^+, 45), 385 (15), 231 (100), 216 (44), 165 (25), 123 (31); IR ($CDCl_3$) 1722 cm^{-1}; analytically calculated for $C_{28}H_{48}O$: C, 83.93; H, 12.07; found: C, 84.05; H, 12.22.

Plant materials and growth conditions. Friable calli were induced from leaves of micropropagated *S. malacoxylon* Sendt. plantlets as described by Suardi *et al.* (34). Cultures of *in vitro* plantlets were maintained under continuous light at 25 C in 0.5 × Gamborg's B5 (35) medium (pH 5.8) supplemented with 1.5% (wt/vol) sucrose, 0.5 × Gamborg's vitamin mixture (35), 0.1 mg/liter indole-acetic acid, 0.08% phytagar. Callus cultures were maintained in the dark at 25 C and subcultured every 3 wk in MS1W medium. MS1W medium was composed as follows: macro- and microelements according to Murashige and Skoog (36), 3% (wt/vol) sucrose; 0.3 mg/liter kinetin; 2 mg/liter 2,4 D, Murashige and Skoog vitamin mixture (36), Gamborg's vitamin mixture, 0.08% phytagar at pH 5.8.

Preparation of vegetal homogenates. Freshly harvested calli and leaf tissues were frozen in liquid nitrogen and stored at −80 C. Frozen specimens were mechanically disrupted on liquid nitrogen using mortar and pestle, and tissue powder was collected in 4 vol 100 mM KH_2PO_4 buffer (pH 7.8) containing 2 mM EDTA, 8 mM $MgCl_2$, 4 mM DTT, 10% glycerol, and 0.1% NOG. Samples were incubated for 30 min on ice after homogenization with vortex and addition of polyvinilpolypirrolidone (1%) to remove phenolic impurities to increase enzymatic stability.

Samples were then centrifuged at 13,500 × *g* at 4 C for 15 min, and total proteins of supernatant were determined by the method of Bradford (37) using BSA as standard.

Determination of 5αR activity in vegetal homogenates. Assays were carried out in a total volume of 1.0 ml containing 250–400 μg proteins in 100 mM KH_2PO_4 buffer (pH 7.4), 1 mM PMSF, and the radioactive steroid precursor (mixture of labeled plus nonlabeled; 0.01–50 μM; 0.2–1 × 10^6 dpm). Assays were performed at 25 C in a shaking water bath (140 rpm) and were initiated by the addition of NADPH to a final concentration of 1 mM. After incubating the reaction mixture for 120 min, the precursor steroid and its metabolites were extracted from the homogenate with 3.0 ml ethyl acetate. The organic fraction was evaporated under a nitrogen stream. After evaporation the residues were dissolved in 100 μl dichloromethane-diethyl ether (85:15 vol/vol), spotted on silica gel plates, and run in the same solvent system. After TLC (UV revelation), the R_f of metabolites were compared with those of standard steroids comigrated on the same plates. The spots were scraped off, silica extracted with 2.0 ml ethyl acetate, and the extract subjected to liquid scintillation counting in the β-counter. The enzymatic activity was calculated from the percent conversion of substrate into product as follows:

C% = [product counts/(substrate counts + product counts)] × 100.

The *in vitro* condition described, incubation time, and protein concentration were chosen within the linear range.

Cell culture and growth conditions. Two transfected cell lines were used for this study: CHO1827 (cells expressing 5αR-1) and CHO1829 (5αR-2) (38).

Transfected CHO cells were maintained in DMEM/F-12 medium (mixture 1:1 of DMEM and Ham's F-12 medium) supplemented with 5% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were maintained in a fully humidified incubator with 95% air and 5% CO_2 at 37 C.

5αR activity assay in cell lysates of CHO1827 and CHO1829. For assay of 5αR activity in cell lysates, cells were harvested from plates in PBS with a rubber policeman, pelleted by centrifugation, and frozen in liquid nitrogen for storage at −80 C or lysed and assayed immediately. Cell pellets were homogenized in 10 mM KH_2PO_4 (pH 7.4), 150 mM KCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 0.1% NOG with three short pulses of a polytron homogenizer (Ultraturrax T8, IKA Labortechnik, Staufen, Germany). The cell lysate protein concentration was determined by the method of Bradford (37) using BSA as standard. 5αR assays were performed in a total volume of 1.0 ml in 10 mM KH_2PO_4 (pH 7.4), 150 mM KCl, 1 mM PMSF, 0.1% NOG, using 10–50 μg proteins. Assays were performed at 37 C in a shaking water bath and initiated by the addition of NADPH to a final concentration of 1 mM. After incubating the reaction mixture for 20 min (substrate progesterone) or 60–90 min (substrate campestenone), the precursor steroids and their metabolites were extracted with 3.0 ml ethyl acetate, and enzyme activity was measured as described above.

5αR activity assay in human prostate homogenates. Frozen prostate tissue was mechanically disrupted on liquid nitrogen using mortar and pestle. Tissue powder was collected in 5 vol 100 mM KH_2PO_4 buffer (pH 7.8) containing 1 mM EDTA, 5 mM DTT, 10% glycerol, and 0.1% NOG and homogenized with three short pulses of the polytron homogenizer. Tissue homogenate was centrifuged at 6000 × *g* at 4 C for 10 min, and the supernatant was assayed for 5αR activity as previously described for cell lysates of CHO cells using 500 μg proteins and an incubation time of 120 min. The concentration of protein in prostate homogenate was determined by the method of Bradford (37) using BSA as standard.

Determination of kinetic constants. Saturation curves were determined using a concentration range 0.01–50 μM of progesterone and 0.01–100 μM campestenone.

Velocities were plotted against substrate concentrations, and the apparent K_m and V_{max} values were calculated using a nonlinear regression procedure based on the Michaelis-Menten equation. An Eadie-Hofstee plot of velocity against velocity over substrate concentration was also used because this plot is reportedly best suited to detect isozyme activities (39). The analysis of data was performed with the computer program GraFit 4.0.16 (Erithacus Software, Horley, Surrey, UK).

Inhibition test. Stock solutions of the inhibitors were prepared at a concentration of 1 mg/ml in ethanol. These solutions were stable for 1 yr after the preparation. Working solutions at concentrations of less than 1 mg/ml were freshly prepared in ethanol.

The concentration range of inhibitor was 0.01–100 μM. The percentage of conversion at each concentration of the inhibitor was normalized to the control (percent of conversion without the inhibitor), and data were processed with the program ALLFIT (40) using the four-parameter logistic equation to calculate the IC_{50} values. This program allowed the statistical analysis of fits from different experiments.

Results

Reduction of campestenone to 5α-campestanone by human 5αR isozymes

Cell lysates, prepared from CHO1827 and CHO1829 cell lines expressing type 1 and type 2 isozymes, respectively, were used to test the ability of human recombinant enzymes to reduce campestenone, the substrate of plant enzyme DET2. Human prostate homogenates were used as source for the native enzymes. Campestenone was reduced to 5α-campestanone by recombinant and native human 5αR isozymes with NADPH as cofactor. Figure 1 shows the Eadie-Hofstee plots obtained for the determination of the kinetic constants for campestenone in transfected CHO cells and human prostate homogenates. The kinetic constants of human recombinant 5αR isozymes for progesterone were also determined as a control. The formation of 5α-reduced steroids in CHO cell lysates was linear from 10 to 50 μg protein and over a 20-min period of incubation with progesterone, and the linearity with the incubation time was maintained over 90 min with campestenone (data not shown).

Table 1 shows the apparent K_m and V_{max} values determined for both recombinant and native isozymes of human 5αR, with campestenone and progesterone as substrates. Steroid 5αR activity was assayed in the presence of 0.01–100 μM campestenone and 0.01–50 μM progesterone. The kinetic constants were calculated using the nonlinear regression procedure based on the Michaelis-Menten equation. The apparent K_m value of recombinant 5αR-1 for campestenone was 25.42 μM. The type 2 recombinant enzyme was found to have a higher affinity for campestenone, with an apparent K_m value of 4.45 μM. The apparent K_m values calculated for progesterone in transfected CHO cells are in agreement with those reported in literature (38). The Eadie-Hofstee plot for

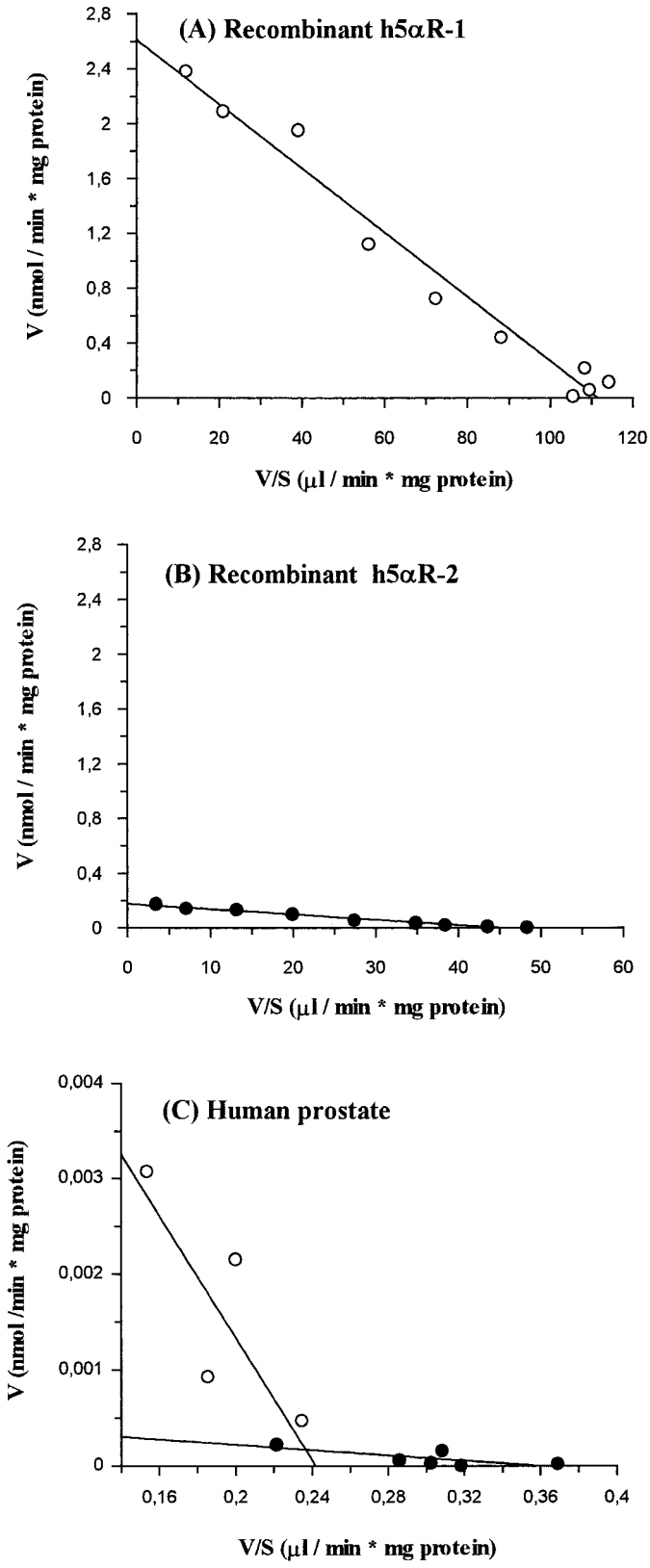

FIG. 1. Eadie-Hofstee plot for (A) recombinant h5αR-1, (B) recombinant h5αR-2, (C) human prostate 5αRs. Velocities were obtained at 0.01–100 μM campestenone and 1 mM NADPH at 37 C. Kinetic constants for these analyses are presented in Table 1.

human prostate homogenate was nonlinear over the whole substrate concentration range showing the presence of two enzyme activities (Fig. 1). The apparent K_m value for 5αR-1 (30.19 μM) and 5αR-2 (1.43 μM) calculated from the Eadie-Hofstee plot in prostate homogenate are comparable with those obtained for recombinant 5αR isozymes.

5αR activity for campestenone in leaves and calli of S. malacoxylon

Two tissues of *S. malacoxylon* were used to test 5αR activity: the leaf as an example of well-differentiated and structured tissue and the callus as a typical undifferentiated plant tissue. Homogenates from these tissues were prepared, and experimental conditions were optimized to maximize the steroid 5αR activity. Total proteins extracted from leaves and calli were incubated with tritium-labeled campestenone in the presence of increasing concentrations of substrate (0.01–10 μM) and 1 mM of NADPH as cofactor. The formation of 5α-reduced steroids was linear from 100 to 500 μg protein and over a 120-min period of incubation (data not shown).

Figure 2 shows the data of a typical experiment for the determination of apparent K_m and V_{max} values for campestenone in callus and leaf homogenates. A Michaelis-Menten relationship between the concentration of substrate and the rate of product formation was observed in both tissues. These data were also analyzed using an Eadie-Hofstee plot of V *vs.* V/S, and both callus and leaf homogenates exhibited linear plots. The kinetic constants for 5αR in calli and leaves of *S. malacoxylon*, summarized in Table 2, were calculated using the nonlinear regression procedure based on the Michaelis-Menten equation. The apparent K_m value calculated in callus homogenates was 0.25 μM and the V_{max} was 1.1 pmol/min per milligram of protein. Surprisingly, the kinetic constants determined in leaves were significantly different, with the K_m being 1.2 μM and the maximum velocity equal to 1.7 pmol/min per milligram of protein. These results suggest that two different isozymes of 5αR could be present in *S. malacoxylon*.

5αR activity for progesterone in leaves and calli of S. malacoxylon

To investigate the biochemical behavior of 5αR in *S. malacoxylon* and point out the similarities with mammalian enzymes, we have used steroid substrates typical of humans. It has been demonstrated that DET2, when transfected in mammalian cells, is able to reduce many substrates of mammalian 5αRs such as testosterone, androstenedione, and progesterone (27). Therefore, we have assayed 5αR activity in *S. malacoxylon* with testosterone, androstenedione, and progesterone; among them, only progesterone was reduced to 5α-dihydroprogesterone. Figure 3 illustrates the results obtained in a typical experiment with homogenates prepared from calli and leaves with progesterone 0.1–20 μM as substrate. Velocities were plotted against progesterone concentration (Michaelis-Menten plot) and against V/S (Eadie-Hofstee transformation). In this case, the Eadie-Hofstee plot for calli was nonlinear over the whole substrate concentration range, showing a different substrate affinity of 5αR at high and low progesterone concentrations. Two 5αR enzyme

TABLE 1. Kinetic constants of human 5α-R isozymes for campestenone and progesterone in cell lysates of CHO1827 and CHO1829 cell lines and human prostate homogenates

Substrate	5αR-1		5αR-2	
	K_m (μM)	V_{max} (nmol/min·mg protein)	K_m (μM)	V_{max} (nmol/min·mg protein)
Campestenone	25.42 ± 2.41[a]	2.70 ± 0.08[a]	4.45 ± 0.42[a]	0.18 ± 0.005[a]
	30.19 ± 13.10[b]	0.008 ± 0.023[b]	1.43 ± 0.44[b]	0.0005 ± 0.001[b]
Progesterone	2.20 ± 0.19[a]	10.82 ± 0.30[a]	0.26 ± 0.03[a]	1.40 ± 0.05[a]

Each K_m value represents the average of at least two experiments carried out in different days. All values are reported as mean ± SD.
[a] Recombinant (5αR in transfected CHO cells).
[b] Native (5αR in human prostate homogenates).

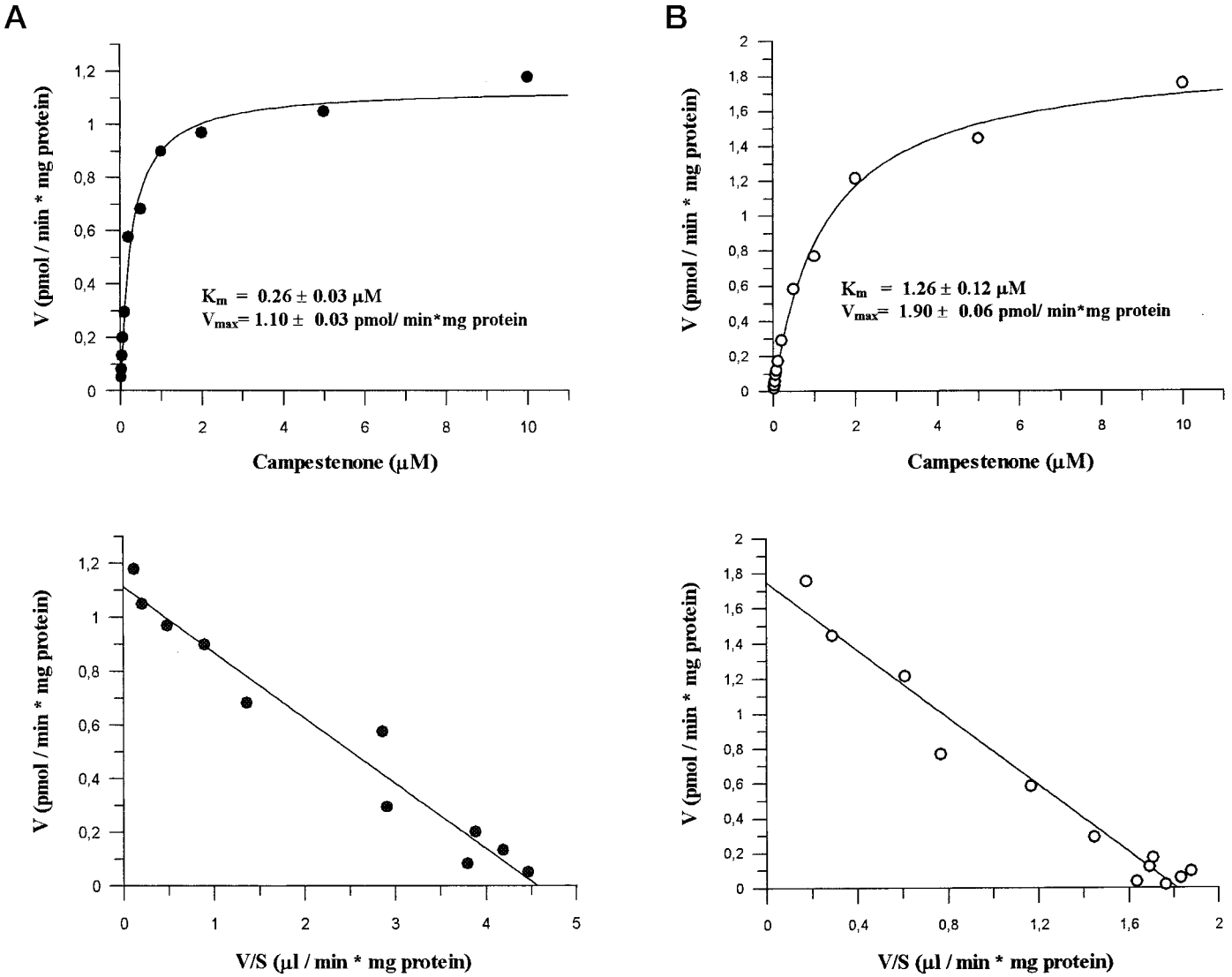

FIG. 2. 5α-reductase activity in homogenates of calli (A) and leaves (B) of *S. malacoxylon*. Velocities were obtained at 0.01–10 μM campestenone and 1 mM NADPH at 25 C. The *solid lines* represent the best fits to the Michaelis-Menten equation (*upper*) and to the Eadie-Hofstee transformation (*lower*). The apparent K_m and V_{max} values, reported as mean ± SD, were calculated by nonlinear regression analysis based on Michaelis-Menten equation. The Eadie-Hofstee plots were linear both in callus and leaf homogenates.

activities with different K_m and V_{max} can be calculated from these data. This suggests that the two isozymes of 5αR may coexist in calli of *S. malacoxylon*.

Table 3 shows the kinetic constants calculated for 5αR in calli and leaves with progesterone. The apparent K_m for 5α-dihydroprogesterone production calculated in leaves was 1.1 μM, and the maximum velocity equal to 6.8 pmol/min per milligram of protein. Two apparent K_m values were calculated from the nonlinear Eadie-Hofstee plot in callus homogenates using the same nonlinear regression procedure based on a Michaelis-Menten equation for two isozymes: a K_m value of 0.06 μM for the enzyme with high affinity for progesterone and a K_m value

of 1.2 μM, similar to that obtained in leaf homogenates, for the enzyme with lower affinity for progesterone.

Effect of inhibitors of human 5αR on the 5α-reduction of progesterone in leaves and calli of S. malacoxylon

To further characterize 5αR activity in calli and leaves of *S. malacoxylon*, some inhibitors of human steroid 5αRs were used. 5αR activity was measured in the presence of finasteride (a specific 5αR-2 inhibitor), LY191704, AS601811, VG106 (three selective 5αR-1 inhibitors), and 4-MA (a dual inhibitor) at the concentration of 10 μM with progesterone 100 nM as substrate. Results are shown in Fig. 4: Finasteride did not affect the 5αR activity both in callus and leaf homogenates, but for the other molecules, the inhibition was higher in leaves than calli. These results support the hypothesis that two different isozymes of 5αR are present in *S. malacoxylon*.

TABLE 2. Kinetic constants of 5α-R activity for campestenone in calli and leaves of *S. malacoxylon*

Tissue	K_m (μM)	V_{max} (pmol/min·mg protein)
Calli	0.25 ± 0.04	1.10 ± 0.06
Leaves	1.20 ± 0.15	1.70 ± 0.05

Each K_m value represents the average of different experiments (n = 6 for calli and n = 4 for leaves) carried out in different days. All values are reported as mean ± SD.

Because 4-MA appeared as the most potent inhibitor in both tissues, we determined the IC_{50} for 4-MA in calli and leaves. The concentration of progesterone was 100 nM and the range of concentrations of 4-MA was 0.01–100 μM. The IC_{50} values were 0.62 μM for 5αR activity in leaf homogenates and 316 μM for 5αR activity in callus homogenates (Fig. 5).

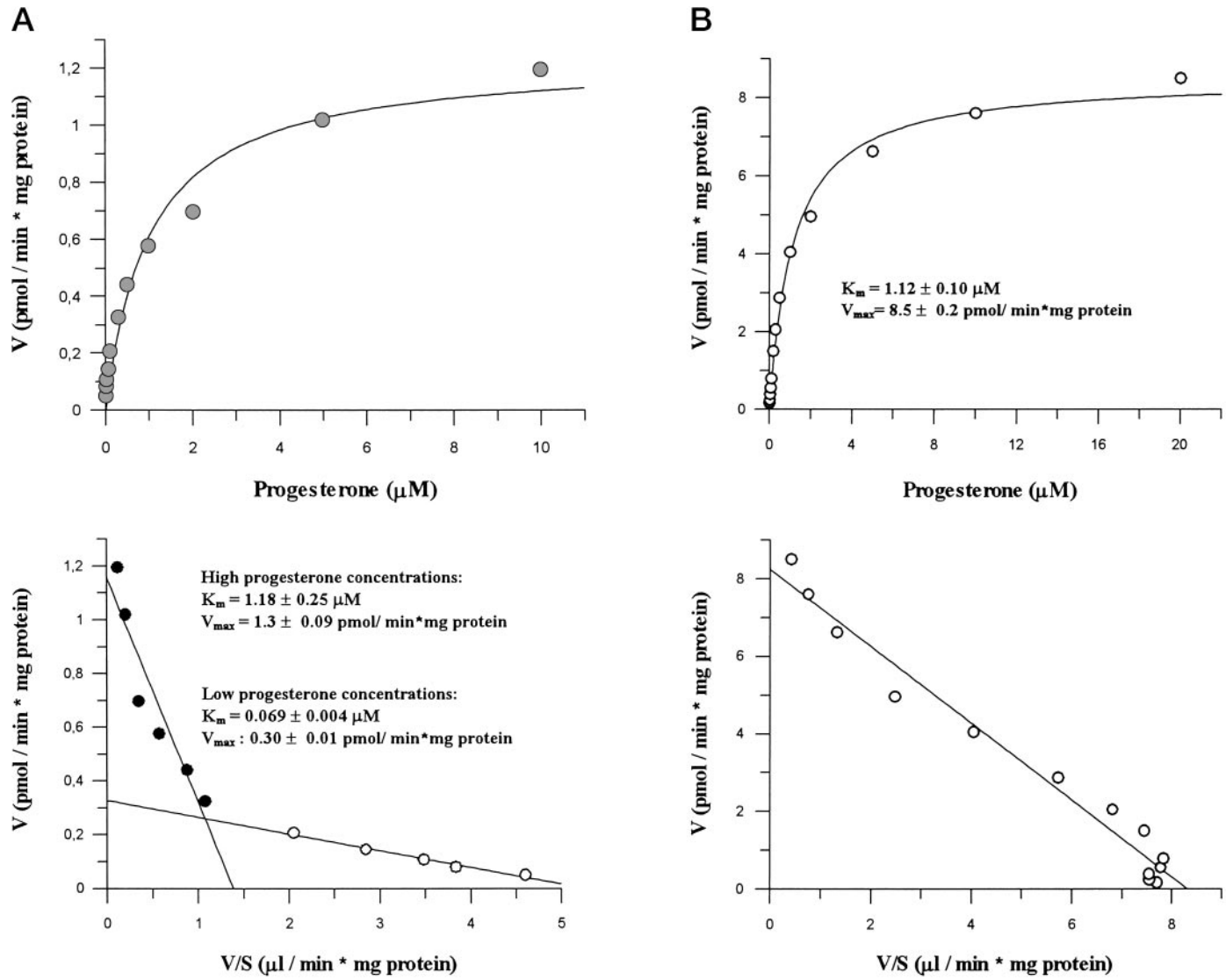

FIG. 3. 5α-reductase activity in homogenates of calli (A) and leaves (B) of *S. malacoxylon*. Velocities were obtained at 0.01–20 μM progesterone and 1 mM NADPH at 25 C. The figure shows the Michaelis-Menten plot (*upper*) and the Eadie-Hofstee transformation (*lower*) for each activity. The kinetic constants of 5α-reductase present in leaves, reported as mean ± SD, were calculated by nonlinear regression analysis based on Michaelis-Menten equation. The Eadie-Hofstee plot of V against V/progesterone concentration is clearly nonlinear in callus homogenates and the kinetic constants of two 5α-reductase isozymes were calculated from these data in calli (mean ± SD).

The IC_{50} values for 4-MA in leaf and callus homogenates were significantly different ($P < 0.01$).

Effect of 4-MA on 5αR activity for campestenone in leaves and calli of S. malacoxylon

The activity of the compound 4-MA was also determined in the presence of campestenone as substrate at the concentration of 10 nM. The IC_{50} value calculated in calli was 5.7 ± 1.2 μM and the IC_{50} value calculated in leaves was 23.9 ± 6.3 μM. The IC_{50} values for 4-MA in leaf and callus homogenates were significantly different ($P < 0.05$), showing again the presence of two 5αRs with different affinities for the same inhibitor.

The pH profiles for 5αR activity for progesterone and campestenone in leaves and calli of S. malacoxylon

The pH profile of 5αR in leaf and callus extracts was determined to establish whether the hypothesized plant isozymes have different pH optima in analogy with the mammalian 5αR isozymes.

5αR activity in leaf and callus homogenates was determined with progesterone and campestenone 100 nM in the pH range of 4.0–9.0 (Fig. 6). With both substrates the pH activity profile for 5αR in calli showed a broad pH optimum that span the alkaline range (pH 7–9), and in the leaf homogenates, 5αR activity had an acidic pH optimum at pH 6.0. This result further supports the hypothesis of the presence of two 5αR isozymes in leaves and calli of *S. malacoxylon.* The two plant isozymes are distinguished by their pH optima as well as mammalian steroid 5αR-1 and -2.

TABLE 3. Kinetic constants of 5α-R activity for progesterone in calli and leaves of *S. malacoxylon*

Tissue	K_m (μM)	V_{max} (pmol/min·mg protein)
Calli	0.063 ± 0.006	0.50 ± 0.02
	1.20 ± 0.25	1.40 ± 0.09
Leaves	1.10 ± 0.19	6.80 ± 0.19

Each K_m value represents the average of four different experiments carried out in different days. All values are reported as mean ± SD.

Discussion

Steroids play a key role in the transmission of signals that mediate growth and physiological responses in most pluricellular organisms. Although the physiological role of steroid hormones in mammals is well known, the role of plant steroid hormones, brassinosteroids, in plant development remains to be fully clarified. Increasing data on brassinosteroids biosynthesis show that the key steps in plant and animal steroid biosynthetic pathway are highly conserved. In this article we focused our attention on the enzyme 5αR, pointing out new similarities between human and plant steroid metabolism.

The ability of the human isozymes of 5αR to reduce campestenone, the natural substrate of the known plant enzyme DET2, is the first direct evidence that the human 5αR system can recognize a plant substrate. The direct determination of the kinetic constants for campestenone shows, in analogy with many human steroid substrates, that the plant substrate is reduced with higher affinity by the type 2 human isozyme than by the type 1.

Campestenone is reduced to 5α-campestanone also in human prostate homogenates, and the determination of the kinetic constants shows the presence of both human 5αR isozymes in this tissue, as demonstrated with human substrates. Campestenone could therefore compete with the human substrates and interfere with the human steroid metabolism.

Because brassinosteroids are hormones found at low levels in many plant tissues, we can suppose that 5αR is equally present in many plant species. In attempting to characterize the 5αR activity in plants, we used *S. malacoxylon* because it is a calcinogenic plant, very active in the biosynthesis of vitamin D-like molecules and sterols. Moreover, information on the biosynthetic pathway of steroids in *Solanum* could be very important because many plants belonging to the same

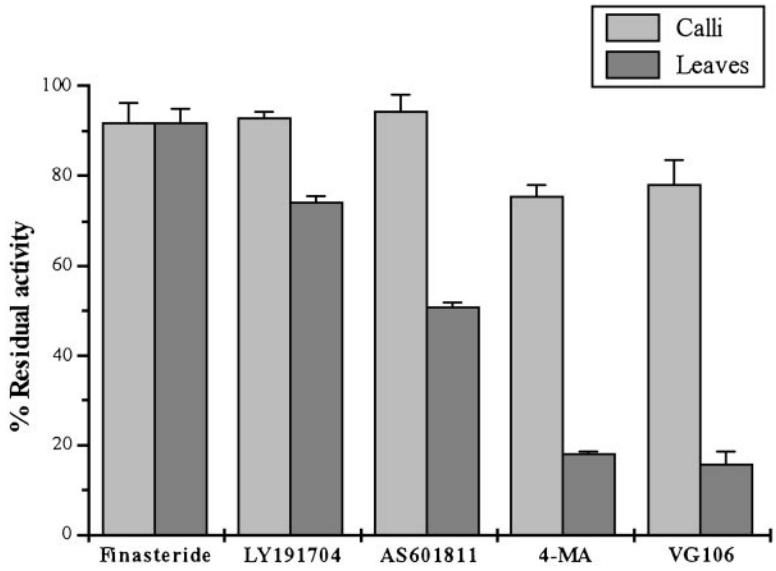

FIG. 4. Inhibition of 5α-reductase in calli and leaves of *S. malacoxylon*. Residual activity (%) is shown at 10 μM concentration of each compound. Each *column* represents mean with SD (n = 2–4).

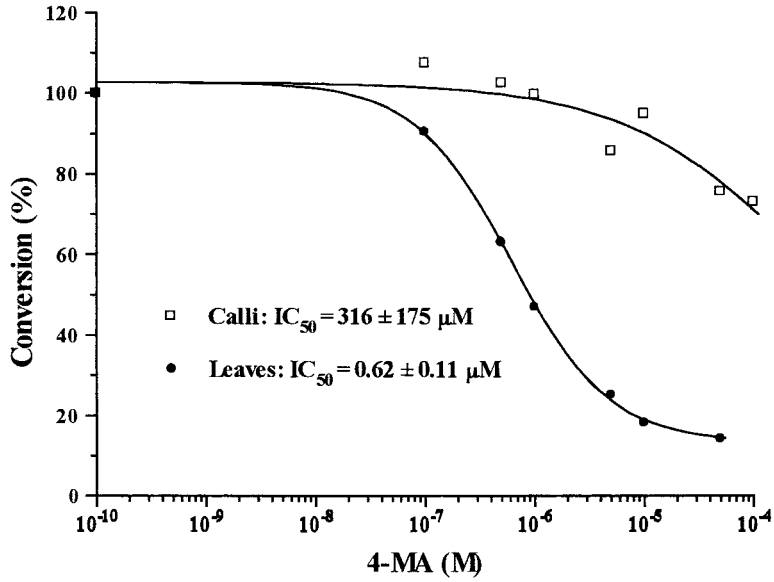

FIG. 5. Inhibition curves of 4-MA toward 5α-reductase present in calli and leaves of *S. malacoxylon*. 5α-reductase activity in callus and leaf homogenates was measured in the presence of increasing concentrations of 4-MA (0.1–100 μM), 100 nM progesterone and 1 mM NADPH at 25 C. The IC_{50} values (mean ± SD) were calculated using the four-parameter logistic equation. The IC_{50} values calculated in leaf and callus homogenates were significantly different ($P < 0.01$).

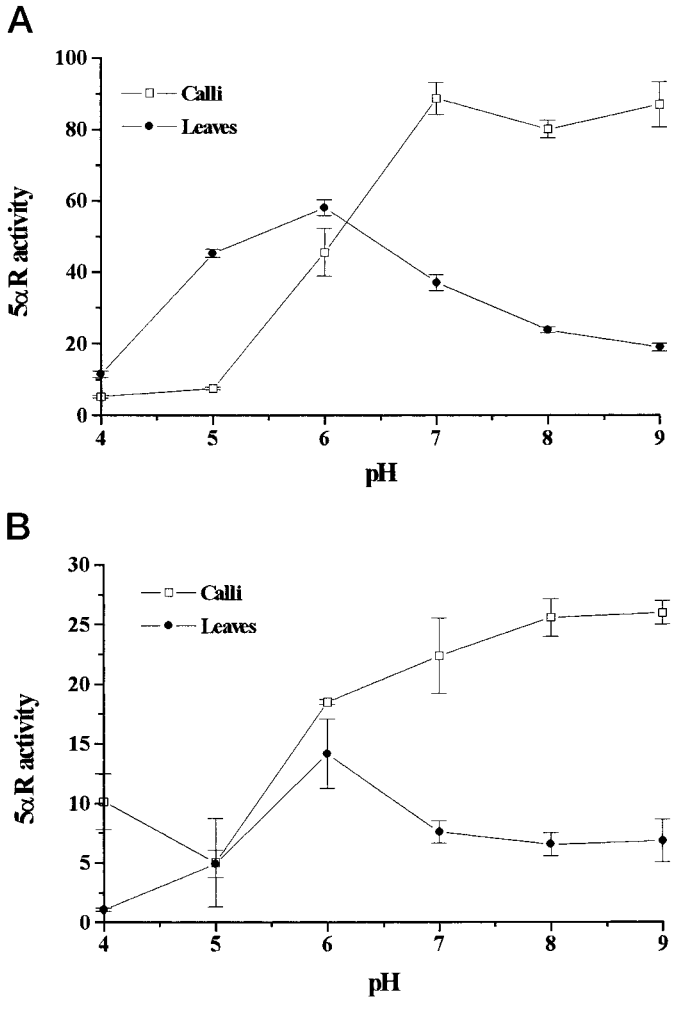

FIG. 6. pH profiles of 5αR activity in callus and leaf homogenates of *S. malacoxylon* in the pH range 4.0–9.0 with 1 mM NADPH as cofactor and 100 nM progesterone (A) and 100 nM campestenone (B) as substrates. 5αR activity is expressed as percent of conversion of substrate into product/mg protein.

family of Solanaceae are common components of the human diet.

To determine whether there are differences of 5αR activity between differentiated and undifferentiated tissues, we decided to study this enzyme in leaves, as an example of a well-differentiated and structured tissue, and in calli, induced from leaves, as an example of undifferentiated tissue. Both these tissues showed 5αR activity using campestenone as substrate. This is the first evidence of the presence of a steroid 5αR enzyme in *S. malacoxylon*. However, the most relevant observation was that the apparent K_m obtained for campestenone in leaves was significantly different from that obtained in calli. These data suggest the presence of different 5αR isozymes in differentiated and undifferentiated tissues. The 5αR found in calli had a higher affinity for the substrate with an apparent K_m 0.25 μM, compared with the 5αR detected in leaves that showed an apparent K_m 1.2 μM. These results extend the analogies between animal and plant enzymatic systems. Mammalian 5αR is in fact a system of two isozymes with different substrate affinity.

In attempting to further characterize the 5αR isozymes in *S. malacoxylon*, we used testosterone, androstenedione, and progesterone as typical substrates of mammalian 5αRs because DET2, when expressed in mammalian cells, was reported to be able to reduce these substrates. Among the tested steroids, only progesterone was reduced in *S. malacoxylon*. Like campestenone, progesterone also showed different apparent K_m in calli and leaves. Moreover, the use of progesterone in calli allowed the detection of both isozymes. The Headie-Hofstee plot of the kinetic data were in fact nonlinear, indicating the coexistence of the two isozymes in the undifferentiated tissue. The determination of the kinetic constants was repeated with four different callus preparations giving very reproducible results. The high-affinity isozyme found in calli showed an apparent K_m 0.06 μM for progesterone. The low-affinity isozyme found in calli and leaves had an apparent K_m 1.1 μM for progesterone. The same callus homogenates, used to de-

termine the kinetic constants for progesterone, were tested with campestenone giving a linear Headie-Hoftee plot with the apparent K_m 0.25 μM. This value can be considered a mean value between the K_m found in leaves and a lower K_m of the high affinity isozyme found in calli. Probably the ratio between the two isozymes does not allow the determination of two distinct K_m values in callus homogenates with campestenone.

In attempting to further characterize the 5αR enzymatic activity in *S. malacoxylon,* we decided to use inhibitors with different potency and selectivity toward the two human 5αR isozymes. The results of inhibition tests, performed with progesterone as substrate, indicated that all the inhibitors were active in plant tissues with the exception of finasteride, in agreement with the observation made by Li *et al.* (27) in mammalian cells transfected with DET2. All inhibitors were more active in leaf homogenates than in callus homogenates. In addition, we determined the IC_{50} of 4-MA, the most potent tested inhibitor, for both the 5αR isozymes of *S. malacoxylon*. The values were 0.62 μM for leaves and 316 μM for calli. The different response to inhibitors in the two tissues supports the hypothesis of the existence of two plant 5αR isozymes and points out the analogies between plant and mammalian 5αRs. The two 5αR systems share the substrates as well as the response to inhibitors, indicating that strong similarities exist in the active sites of all the 5αRs. The IC_{50} of 4-MA was determined with campestenone as substrate, and in this case we also found differences between calli and leaves; however, the inhibitor was more active in callus homogenates (IC_{50} = 5.7 μM) than in leaf homogenates (IC_{50} = 23.9 μM).

The pH profile of 5αR activity in callus and leaf extracts indicates another similarity between plant and mammalian 5αRs. Callus homogenates showed a broad pH optimum in the alkaline range 7–9, and leaf homogenates had the maximum of 5αR activity at pH 6. In humans the pH optima for the two 5αR isozymes are 6–8.5 for 5αR-1 and 5.5 for 5αR-2, respectively.

In conclusion, the data presented in this article demonstrate for the first time the existence of a 5αR activity in *S. malacoxylon*, a plant belonging to the Solanaceae. The Solanaceae family includes many plants widely used as food (tomatoes, potatoes, eggplants, peppers) and commercially important plants (tobacco and medicinal plants). The presence of two isozymes in different plant tissues of *S. malacoxylon* highlights the parallelism, evidenced also by other authors, between plant and mammalian steroid metabolic pathway. Finding that the plant and human enzymatic systems share the same substrates and inhibitors could open important possibilities of application. Plant molecules that could regulate the plant steroid biosynthesis could interact, through the diet, with the human steroid metabolism. On the other hand, the knowledge on the relationship between structure and activity of human enzyme inhibitors could be applied to design molecules active on the plant steroid metabolism.

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