The Prostate 13:289–297 (1988)

Effect of a 4-Methyl-4-Aza Steroid on Androgen Metabolism by Rat Ventral Prostate Epithelial and Stromal Cell Cultures: Selective Inhibition of 5α-Reductase Activity

John Orlowski and Albert F. Clark

Departments of Biochemistry (J.O., A.F.C.) and Pathology (A.F.C.), Queen's University and Kingston General Hospital, Kingston, Ontario, Canada

The effect of a potent steroid metabolic inhibitor, 17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (DMAA), on androgen metabolism was investigated in primary monolayer cultures of rat ventral prostate epithelial and stromal cells. Using testosterone (T) as substrate, 5α-reductase (5α-R) activity in both cell types was inhibited by greater than 98% at an inhibitor concentration of 1000 nM. The concentrations required to produce a 50% inhibition (IC_{50}) were 7.4 and 9.0 nM for epithelial and stromal cells, respectively. To examine the specificity of this compound, its effect on other steroid-metabolic enzymes was examined. DMAA at a concentration of 1,000 nM had no effect on 3α-hydroxysteroid oxidase (3α-HSORox), 3-ketosteroid reductase (3α-HSORred), and 6/7-hydroxylase (6/7-HSH) activities in both cell types; 17β-hydroxysteroid oxidase (17β-HSORox) activity, located primarily in epithelial cells, also was not influenced by DMAA. In contrast, epithelial 3β-hydroxysteroid oxidase (3β-HSORox) and 3-ketosteroid reductase (3β-HSORred) activities were inhibited by 65% ($P < .001$) and 58% ($P > .05$), respectively, albeit the latter result was not statistically significant. Stromal 3β-HSORox and 3β-HSORred activities were negligible; hence the effect of the inhibitor on these enzymes could not be assessed. In conclusion, DMAA is a relatively selective and potent inhibitor of 5α-R activity in primary cultures of rat ventral prostate epithelial and stromal cells and should be a useful compound for antagonizing androgen-mediated actions in the prostate and other androgen target tissues.

Key words: rat prostate, cell cultures, DMAA, 5α-reductase

INTRODUCTION

Androgen-mediated actions in the prostate are dependent on the formation of 5α-dihydrotestosterone (5α-DHT) [1]; 5α-DHT is the androgen which binds to the androgen receptor. In vivo, 5α-DHT formation occurs mainly by the irreversible

Received for publication July 27, 1987; accepted September 9, 1988.

Address reprint requests to Albert F. Clark, Department of Biochemistry, Queens University, Kingston, Ontario K7L 3N6, Canada.

John Orlowski is now at the Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, ML 524, Cincinnati, OH 45267.

reduction of testosterone (T) by the enzyme Δ^4-5α-reductase (5α-R); 5α-R activity can therefore play a key role in the regulation of prostate growth and function [2].

In recent years there has been considerable evidence from tissue recombinant experiments demonstrating an important role for epithelial-stromal cellular interactions in rat prostate morphogenesis, growth, and cytodifferentiation [3,4]. These studies indicate that the presence of the stroma is required for the development and maintenance of the rodent urogenital sinus epithelium. The stroma requires the presence of androgen, or more specifically 5α-DHT, for this inductive effect. A similar intercellular interrelationship has been suggested for the human prostate [5,6]. For example, human prostate epithelial-stromal cellular interactions have implicated the androgen-dependent stroma in the pathogenesis of human benign prostate hyperplasia (BPH) [5]. Human BPH stroma has been reported to have elevated levels of 5α-R activity compared to normal stroma [7]. Whether this leads to elevated prostate 5α-DHT concentrations is unclear. Also, it is not known whether 5α-DHT plays a casual or permissive role in the development of BPH. Regardless of its role in the disease process, reduction in the levels of intracellular prostatic 5α-DHT, particularly in the stroma, should reduce or prevent further abnormal growth of androgen-sensitive prostate tissue. This suggests that 5α-R should be a reasonable target for therapeutic agents.

Recently a 4-methyl-4-aza steroid, 17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (DMAA; also referred to as 4-MA), has been shown to be a potent inhibitor of 5α-R activity both in vivo [8,9] and in vitro [10,11] as well as being an inhibitor of prostate growth. To date, no studies have been reported on the direct effect of DMAA on androgen metabolism by separated prostate epithelial and stromal cells. Understanding the effectiveness of DMAA in inhibiting androgen actions in both cell types may be important to understanding its role as a therapeutic agent. This report examines the effects of DMAA on androgen metabolism in primary cultures of ventral prostate epithelial and stromal cells derived from immature rats.

MATERIALS AND METHODS

Animals

Experiments were performed by using immature male Sprague-Dawley rats (21–22 d old) obtained from Canadian Breeding Farms and Laboratories Ltd. (Montreal, Quebec). All animals were housed in an animal room with a controlled environment and a regulated light cycle (14-h light : 10-h darkness). Rats were fed tap water and Purina Rat Chow ad libitum.

Steroids

[1,2-^{3}H]T (59 Ci/mmol), [1,2-^{3}H] 5α-DHT (59 Ci/mmol), [1,2-^{3}H] 5α-androstane-3α, 17β-diol (3α-Adiol) (30.1 Ci/mmol), [4-^{14}C]T(57.5 mCi/mmol), and [4-^{14}C]5α-DHT (57.5 mCi/mmol) were obtained from New England Nuclear Corp. (Lachine, Quebec) and were purified prior to use by paper chromatography as described by Bush [12]. [4-^{14}C]3α-Adiol was prepared in our laboratory from [4-^{14}C]5α-DHT by using rat lung cytosol as the source of 3α-hydroxysteroid oxidoreductase. [1,2^{3}H] 5β-androstane-3β,17β-diol (3β-Adiol) and [4-^{14}C]3β-Adiol were prepared from the corresponding labeled 5α-DHTs by reduction with $NaBH_4$ as

previously described [13]. Both ^{14}C- and ^{3}H-labeled 3α- and 3β-Adiol were purified by thin-layer chromatography on silica gel G in chloroform:methanol (98.2) followed by paper chromatography in the Bush B3 system. All radiolabeled steroids were at least 97% pure as determined by thin-layer and paper chromatography. [^{14}C]-steroid recovery standards were prepared in methanol and contained 5,000 dpm of the [^{14}C]-steroid and 25 μg radioinert steroid per 100 μl. Radioinert steroids were obtained from Steraloids, Inc. (Wilton, NH) and Sigma Chemical Co. (St. Louis, MO). DMAA was obtained through the courtesy of Dr. E.H. Cordes, Merck, Sharp & Dohme Research Laboratories (Rahway, NJ).

Beef pancreatic DNase I collagenase, type IV (140 units/mg), Hepes, insulin, transferrin, retinoic acid, dexamethasone, and highly polymerized calf thymus DNA were obtained from Sigma Chemical Co. (St. Louis, MO). Percoll was purchased from Pharmacia Fine Chemicals (Montreal, Quebec). Fetal bovine serum, chicken serum, trypan blue exclusion dye, F12 medium, and DME medium without sodium bicarbonate were purchased from Flow Laboratories Ltd. (Mississauga, Ontario). Lyophilized penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), fungizone (250 g/ml), trypsin (1:250 "Difco" certified), and 0.05% trypsin:0.02% EDTA solution were obtained from Grand Island Biological Co. (New York, NY). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific Co. Hoechst 33258 stain was purchased from Calbochem-Behring (La Jolla, CA).

Cell Separation and Culture of Epithelial and Stromal Cells

Ventral prostates were aseptically removed from immature rats, minced, and dissociated into cells with a mixture of collagenase, trypsin, and chicken serum as previously described [14,15]. The cells were then separated into epithelial- and stromal-cell-enriched fractions by using preformed, continuous isopycnic Percoll gradients [14] with modifications described previously [15]. The cells were cultured in F12/DME medium containing 10% fetal bovine serum, 20 mM Hepes, 10 mM $NaHCO_3$, insulin (5μg/ml), transferrin (5μg/ml), 50 nM T, penicillin (100 U/ml), streptomycin (100 g/ml), and fungizone (1 g/ml). The medium for the epithelial cells also contained 50 nM dexamethasone and 500 nM retinoic acid. Other culture details are given in reference [15]. Cultures reached confluency in 5–7 d. Cells were monitored daily and photomicrographs were prepared by using a Leitz (Wetzlar) inverted phase-contrast light microscope. Representative cultures used for photomicrographs were fixed in 100% methanol for 5 min and stained with 2% Giemsa stain for 5–30 min.

Studies on Androgen Metabolism

Androgen metabolism was studied in confluent cultures as previously described [15]. The culture medium was removed from confluent cultures and replaced with serum-free and steroid-free medium for 24 h. The medium was then replaced with fresh serum-free F12/DME medium containing 50 nM ^{3}H-androgens and varying concentrations of DMAA. Following a further 6 h in culture, medium and cells were removed and extracted with methylene chloride; ^{14}C-labelled steroids were added prior to extraction to monitor losses. The extracts were filtered through anhydrous sodium sulphate and evaporated to dryness under nitrogen. The labelled steroids in the extracts were analyzed by utilizing HPLC. The direct extracts were chromato-

TABLE I. Summary of Steroid Activities Assayed Indicating the Substrates Used and the Metabolites Measured

Steroid enzyme activity	^{3}H-substrate (50 nM)	Metabolites summed (+) to calculate total steroid enzyme activity						
		5α-DHT	3α-Adiol	3β-Adiol	5α-A	An	IsoAn	5α-$C_{19}O_3$
5-R	T	+	+	+	+	+	+	+
3α-HSORox	3α-Adiol	+	−	+	+	−	+	+
3β-HSORox	3β-Adiol	+	+	−	+	+	−	−
17β-HSORox	5α-DHT	−	−	−	+	+	+	+[a]
3α-HSORred	5α-DHT	−	+	−	−	+	−	−
3β-HSORred	5α-DHT	−	−	+	−	−	+	+
6/7-HSH	3β-Adiol	−	−	−	−	−	−	+

[a]Refers only to 17-oxo-5α-$C_{19}O_3$ metabolites (i.e., 6α- and 7α-Adiol-17-one).

graphed on a Beckman reverse-phase ultrasphere-ODS column (4.6 min I.D. × 250 mm) with 5-μm spherical packing by using an isocratic mobile phase of acetonitrile-methanol-water (1:3:3) [16]. ^{3}H and ^{14}C in the effluent were monitored and measured with a Flo-one/Beta radioactivity continuous flow detector (model IC; Radiomatic Instruments and Chemical Co. Inc., Tampa, FL). The ^{3}H counts in the peaks were corrected for procedural losses by using the ^{14}C counts. This column system separates T, androstenedione, and their 5α-reduced products [5α-DHT, 3α-Adiol, 3β-Adiol, 5α-androstane-3, 17-dione, androsterone (5α-androstane-3α-ol-17-one), isoandrosterone (5α-androstane-3β-ol-17-one), and several hydroxlyated products. Based on the work of others [17,18], the hydroxylated products are presumed to be hydroxylated at the 6 and 7 positions. By studying the metabolism of T, 5α-DHT, 3α-Adiol, and 3β-Adiol one obtains measures of 5α-R and 3α- and 3β-hydroxysteroid oxidoreductases in the oxidative (3α-HSORox and 3β-HSORox) and reductive (3α-HSORred and 3β-HSORred) and 17β-hydroxyesteroid oxidoreductase in the oxidative direction (17β-HSORox) and hydroxylase (6/7-HSH) activities. Table I lists the enzyme activities measured and indicates the substrates utilized and the metabolites measured. Enzyme activities are expressed as picomoles metabolites formed/6 h/μg DNA. DNA concentrations were measured by the method of Labarca and Paigen [19] by utilizing the enhancement of fluoresence of a bisbenzimide compound (Hoechst 33258 stain). Statistical analysis was performed by using the Student's t-test ($n \leq 30$).

RESULTS

Cell Culture Characteristics

Light microscope photomicrographs of primary monolayer cultures of rat ventral prostate epithelial and stromal cells are presented in Figure 1a and 1b, respectively. Morphologically, the epithelial cells are polygonal in shape and closely associated with each other, which is characteristic of this cell type in culture. In contrast, the stromal cells are elongated, spindle-shaped, and loosely associated with each other. This is characteristic of fibroblast-type cells in culture. The appearance of both cell types was similar whether they were grown in the absence or presence of T. A previous study [15] has demonstrated that both cell types retain differentiated cell

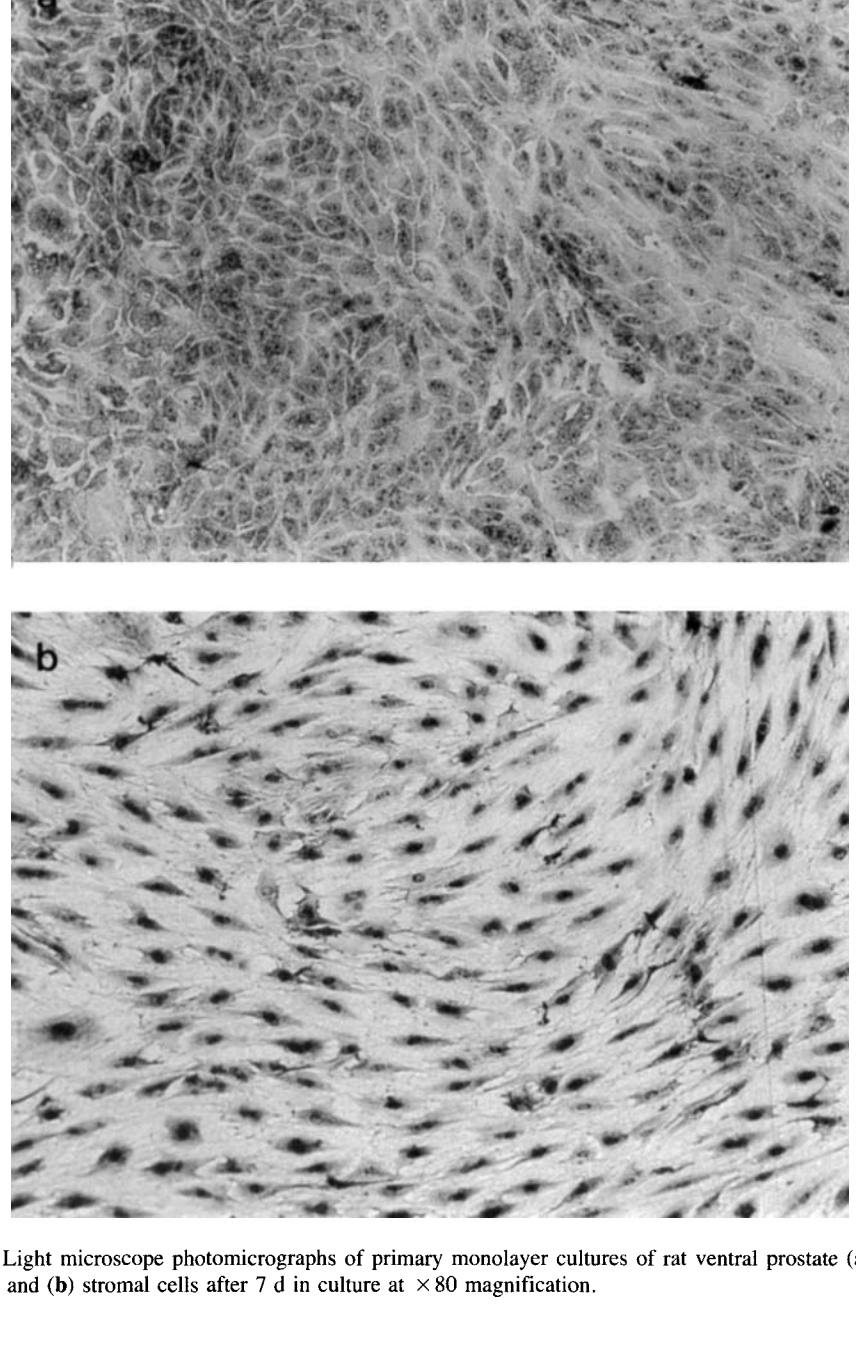

Fig. 1. Light microscope photomicrographs of primary monolayer cultures of rat ventral prostate (**a**) epithelial and (**b**) stromal cells after 7 d in culture at $\times 80$ magnification.

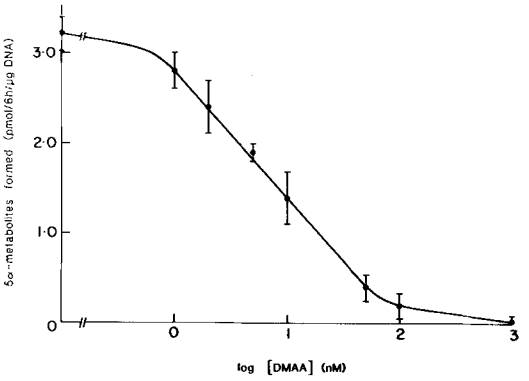

Fig. 2. Effect of DMAA concentration on 5α-reductase activity in rat ventral prostate epithelial cell culture. Results are expressed as the mean ± SD (n = 3).

functions characteristic of the prostate, i.e., the ability to metabolize androgens. The epithelial cells also synthesize secretory acid phosphatase.

Effect of DMAA Concentration on 5α-Reductase Activity

To assess the effect of DMAA concentration on 5α-R activity, confluent monolayer cultures of rat ventral prostate epithelial and stromal cells were co-incubated with [^{3}H]T (50 nM) and increasing concentrations of DMAA (1–1,000 nM); 5α-R activity was expressed as picomoles 5α-reduced metabolites formed from T per 6 h per μg DNA. The inhibition curves for epithelial and stromal cell cultures are shown in Figures 2 and 3 respectively. In both cell types, prostatic 5α-R activity was inhibited by greater than 98% at an inhibitor concentration of 1,000 nM. The concentrations required to produce 50% inhibition (IC_{50}) were 7.4 nM and 9.0 nM for epithelial and stromal cells, respectively.

Effect of DMAA on Other Prostate Steroid-Metabolizing Enzyme Activities

To further investigate the effect of DMAA on other enzymes involved in androgen metabolism by primary cultures of rat ventral prostate epithelial and stromal cells, 50 nM ^{3}H-androgens (T, 5α-DHT, 3α-Adiol, and 3β-Adiol) were incubated separately in the absence and presence of a single concentration of DMAA (1,000 nM). The distribution of androgen metabolites was analyzed following a 6-h incubation period and relative enzyme activities were determined.

The results are presented in Table II. DMAA had some effect on epithelial 3β-HSORox and 3β-HSORred activities. The 3β-HSORox and 3β-HSORred were inhibited by 65% ($P < .001$) and 58% ($P > .05$), respectively, albeit the latter effect was not statistically significant. DMAA had no effects on other major androgen-metabolizing enzymes—3α-HSORox, 3α-HSORred, or 6/7-HSH in both cell types or 17β-HSORox in the epithelial cells.

DISCUSSION

Our laboratory has been developing primary cultures of rat ventral prostate epithelial and stromal cells as a model system for studying androgen-mediated actions

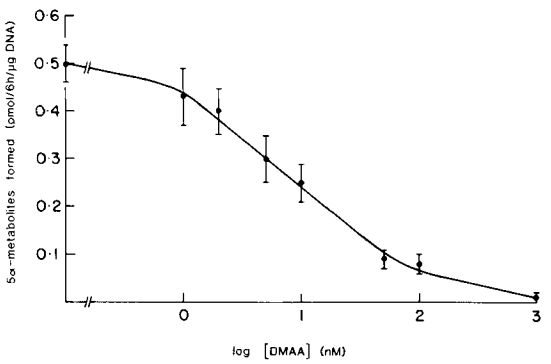

Fig. 3. Effect of DMAA concentration on 5α-reductase activity in rat ventral prostate stromal cell cultures. Results are expressed as the mean ± SD (n = 3).

TABLE II. Effect of DMAA on Steroid-Metabolic Enzyme Activities in Rat Ventral Prostate Epithelial and Stromal Cell Cultures

		Epithelial		Stromal	
Steroid enzyme	Substrate	−DMAA	+DMAA	−DMAA	+DMAA
5α-R	T	3.22 ± 0.44	ND	0.52 ± 0.10	ND
3α-HSORox	3α-Adiol	5.34 ± 0.07	5.42 ± 0.07	4.63 ± 0.68	3.80 ± 0.60
3β-HSORox	3β-Adiol	0.80 ± 0.10	0.28 ± 0.07*	ND	ND
3α-HSORred	5α-DHT	0.15 ± 0.04	0.16 ± 0.09	0.13 ± 0.06	0.20 ± 0.10
3β-HSORred	5α-DHT	0.24 ± 0.04	0.14 ± 0.08	ND	ND
17β-HSORox	5α-DHT	2.39 ± 0.25	2.66 ± 0.32	ND	ND
6/7 -HSH	3β-Adiol	3.83 ± 0.29	4.34 ± 0.20	12.36 ± 0.27	11.95 ± 0.59

Enzyme activities were determined as described in Table I and are expressed as picomoles metabolites formed/6 h/μg DNA. Results are expressed as the mean ± SD (n = 3). ND = not detected.
*$P < .001$.

in the prostate. The relevance of using this system for preliminary investigation on the effectiveness of DMAA as a potential therapeutic agent for humans is supported by the recent finding that this inhibitor is equipotent in inhibiting 5α-R activity in rat and human prostate homogenates (K_i; 5.3 nM and 8.3 nM, respectively) but only 5% as potent an inhibitor of the canine enzyme [20]. The apparent K_m values of T for prostatic 5α-R in rats and humans are similar, being 2.4 μM and 3.3 μM, respectively [21]. Assessing the effectiveness of DMAA in epithelial and stromal cell cultures is important for a number of reasons: 1) both cell types can form 5α-DHT and 2) both cell types are androgen-sensitive and appear to make interactive contributions to overall prostate growth and function.

The results of this study indicate that inhibition of 5α-reduced metabolite formation from T was directly proportional to the concentration of DMAA in both cell types. The concentration of DMAA needed to inhibit formation of 5α-reduced metabolites from T by 50% (IC_{50}) was similar in both cell types (7.4 nM and 9.0 nM, respectively). This suggests that the 5α-R enzymes in both cell types are similar. The IC_{50} values are very similar to the reported apparent K_i values (K_i, 5.3 nM) of DMAA for rat prostatic 5α-R with whole tissue. In contrast to the inhibition of 5α-R activity, DMAA has no effect on the other steroid-metabolic enzyme activities with the exception of 3β-HSOR oxidative and reductive activities. The effect on the latter

enzymes in the prostate must be considered of lesser importance because even at the high DMAA concentration used, the 3β-HSOR activities were inhibited by only approximately 60%.

A previous study [10] using whole tissue preparations of rat ventral prostate has reported that DMAA has two modes of action. It has a high affinity for 5α-R (K_i, 5.3 nM) and a moderate affinity for the androgen receptor (K_d, 3.0 μM). The apparent K_m value of T for rat prostatic 5α-R is 2.4 μM and the affinity of DHT for the androgen receptor is 3.0 nM. Thus DMAA has a 500-fold greater affinity for 5α-R than T but a 1,000-fold lower affinity for the androgen receptor than 5-DHT. DMAA was shown to be a competitive inhibitor with T for 5α-R [10]. There are reports that DMAA inhibits seminal vesicle and prostate growth in 5α-DHT-replaced immature castrate rats [8] and PC-82 tumor (human androgen-dependent tumor line) growth in both T- and 5α-DHT-replaced mice [20]. The authors of the latter paper raised the possibility of a DMAA metabolite competing for the androgen receptor in view of the low affinity of DMAA itself for the receptor. They [20] also raise the possibility of a direct tumoricidal effect of DMAA.

The effectiveness of DMAA as an antiandrogen has been confirmed in recent in vivo studies in rats [8] and dogs [9]. In both species, high pharmacologic concentrations of DMAA administered to intact animals decreased total intracellular androgen levels as well as prostatic weight.

In conclusion, the present study confirms and extends previous studies using whole prostate tissue preparations [8–10,21] to show that DMAA is a strong, relatively specific inhibitor of 5α-R activity in primary cultures of rat ventral prostate epithelial and stomal cells. Furthermore, the results confirm the use of this cell culture system as a preclinical model for drug evaluation. The effectiveness of this antiandrogen in antagonizing androgen-dependent parameters such as epithelial secretory function has been demonstrated (unpublished results). Its effectiveness as an inhibitor of epithelial and stromal cell proliferation in culture is being investigated.

ACKNOWLEDGMENT

The work described in this paper was supported in part by the Medical Research Council of Canada (grant MT-2338).

REFERENCES

1. Mooradian AD, Morley JE, Korenman SG: Biological actions of androgens. Endocr Rev 8:1–28, 1987.
2. Tenniswood M, Bird CE, Clark AF: The role of androgen metabolism in the control of androgen action in the rat prostate. Mol Cell Endocrinol 27:89–96, 1982.
3. Cunha GR, Chung LWK, Shannon JM, Reese BA. Stromal-epithelial interactions in sex differentiation. Biol Reprod 22:19–42, 1980.
4. Lasnitski J, Mizuno T: Role of the mesenchyme in the induction of the rat prostate gland by androgens in organ culture. J Endocrinol 82:171–178, 1979.
5. McNeal JE: Origin and evolution of benign prostatic enlargement. Invest Urol 15:340–345, 1978.
6. Franks LM, Riddle PN, Carbonell AW, Gey GD: A comparative study of the lack of growth capacity of adult human prostatic epithelium mechanically separated from its stroma. J Pathol 100:113–119, 1970.
7. Wilkin RP, Bruchovsky N, Shnitka TK, Rennie PS, Conneau TL: Stromal 5α-reductase activity is elevated in benign prostatic hyperplasia. Acta Endocrinol (Copenh) 94:284–288, 1980.

8. Brooks JR, Baptista EM, Berman C, Ham EA, Hickens M, Johnston DBR, Primka RL, Rasmusson GH, Reynolds GF, Schnitt SM, Arth GE: Response of rat ventral prostate to a new and novel 5α-reductase inhibitor. Endocrinology 109:830–836, 1981.
9. Brooks JR, Berman D, Glitzer MS, Gordon LR, Primka RL, Reynolds GF, Rasmusson GH: Effect of a new 5α-reductase inhibitor on size, histologic characteristics, and androgen concentrations of the canine prostate. The Prostate 3:35–44, 1982.
10. Liang T, Heiss CE: Inhibition of 5α-reductase receptor binding and nuclear uptake of androgens in the prostate by a 4-methyl-4-aza steroid. J Biol Chem 256:7998–8005, 1981.
11. Liang T, Rasmusson GH, Brooks JR: Biochemical and biological studies with 4-aza-steroidal 5α-reductase inhibitors. J Steroid Biochem 19:385–390, 1983.
12. Bush IE: Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem J 50:370–378, 1952.
13. Van Doorn EJ, Burns B, Wood D, Bird CE, Clark AF: *In vivo* metabolism of ^{3}H-dihydrostestosterone and ^{3}H-androstanediol in adult male rats. J Steroid Biochem 6:1549–1554, 1975.
14. Orlowski J, Bird CE, Clark AF: Preparation of epithelial and stromal cell fractions from immature rat prostatic tissue using Percoll gradients. J Androl 3:232–240, 1982.
15. Orlowski J, Clark AF: Androgen metabolism and actions in rat ventral prostate epithelial and stromal cell cultures. Biochem Cell Biol 64:583–593, 1986.
16. Orlowski J, Clark AF: An isocratic reverse-phase high performance liquid chromatography system for analysis of 5α-reduced androgen metabolites. Unpublished data.
17. Isaacs JT, McDermott JR, Coffey DS: The identification and characterization of a new $C_{19}O_3$ steroid metabolite in the rat ventral prostate: 5α-androstane-3β,6α,17β-triol. Steroids 33:639–657, 1979.
18. Ofner P, Vena RL, Leav I, Hamilton DW: Metabolism of C_{19}-radiosteroids by explants of canine prostate and epididymis with disposition as hydroxylated products: A possible mechanism for androgen inactivation. J Steroid Biochem 11:1367–1379, 1979.
19. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102:344–352, 1980.
20. Androle GL, Rittmaster RS, Lariaux DL, Kish ML, Linehan WM: The effect of 4MA, a potent inhibitor of 5 alpha-reductase, on the growth of androgen-responsive human genitourinary tumors grown in athymic nude mice. The Prostate 10:189–197, 1987.
21. Liang T, Casciera MA, Cheung AH, Reynolds GF, Rasmusson GH: Species differences in prostatic steroid 5α-reductases of rat, dog and human. Endocrinology 117:571–579, 1985.