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SELECTIVE INHIBITION OF THE 5α-REDUCTASE OF THE RAT EPIDIDYMIS

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Summary—The effect of several synthetic steroids belonging either to the 4-aza-3-oxo-steroid family or to androstene and androstane derivatives was investigated "*in vitro*" on the epididymal as well as prostatic 5α-reductase activity. For this purpose rat caput epididymis and prostate were incubated with the different steroidal compounds at molar concentrations of 10^{-7}, 10^{-6} and 10^{-5} in the presence of labelled testosterone as substrate. The steroids 4-MA (17β, *N*,*N*-diethyl-carbamoyl-4-aza-5α-androstan-3-one) and 4-OH-A (4-hydroxy-androstenedione), already known to be effective 5α-reductase inhibitors at the level of the prostate, have been used as reference molecules. The 5α-reductase activity was evaluated by measuring pg of dihydrotestosterone (DHT) formed in 2 h of incubation by mg of tissue. The steroids **A**, **B**, **C**, **F**, **G** and **I** inhibit the formation of DHT in the rat epididymis although to different extents; they are also equally effective on the formation of DHT in the rat prostate. The steroids **D**, **E**, **H** and **L** are devoid of any inhibitory property on the formation of DHT in both the rat epididymis and prostate. The most interesting results were obtained with compound **M** which exhibits a dose-dependent and significant inhibitory effect on the formation of DHT in the epididymis, but it is inactive at the level of the prostate. These findings suggest that it is possible (a) to selectively interfere with the 5α-reductase of the epididymis without affecting that present in the prostate, and (b) consequently to envisage new ways to regulate male fertility.

INTRODUCTION

In the epididymis, as in the majority of androgen-dependent structures, testosterone undergoes extensive enzymatic transformations (see [1] for references). This organ is able to metabolize testosterone into 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT) in high yields through the action of the 5α-reductase. This enzyme has recently been cloned [2,3]. DHT may be further metabolized to 5α-androstan-3α, 17β-diol (3α-diol) as well as to 5α-androstan-3β, 17β-diol (3β-diol) through the action of two 3α- and 3β-hydroxysteroid dehydrogenases (see [1] for references).

In general, the 5α-reduced metabolites of testosterone act as the intracellular mediators for many of the multiple actions the hormone exerts on its target structures (see [4, 5] for references). In particular, in the epididymis the 5α-reduced metabolites of testosterone appear to be responsible for both the growth and the function of this structure as well as for the maturation and storage of the spermatozoa [6, 7]. After castration, the fertilizing capacity of the epididymal spermatozoa can be maintained only after substitution therapy performed with either testosterone, DHT or 3α-diol [8]. In addition, the administration of 5α-reductase inhibitors to castrated testosterone-pretreated mice induces a decrease of the "*in vitro*" fertilizing capacity of the spermatozoa as well as a decrease in sperm number and mobility [9]. These data may indicate that DHT and 3α-diol are the predominant steroids involved in the control of the process of maturation of the spermatozoa; this also appears to be true in humans [10]. Consequently, selective inhibition of the conversion of testosterone to DHT would attenuate the responses of the hormone attributable to DHT and its metabolites; this will prevent the final maturation of the spermatozoa.

The purpose of the present study was to investigate the possibility of selectively inhibiting the 5α-reductase present in the epididymis. For this, the effect of several synthetic steroids on the epididymal 5α-reductase activity was

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investigated "*in vitro*". Rat prostate, which is known to be rich in 5α-reductase activity (see [5] for references), was used as control tissue. 4-MA (17β,*N*,*N* diethyl-carbamoyl-4-methyl-4-aza-5α-androstan-3-one) and 4-OH-A (4-hydroxy-androstenedione), two steroids already known as effective 5α-reductase inhibitors at the level of the prostate [11, 12], have been used for comparison. Among the compounds tested 17β-methoxy-17-methyl-(5α)-1H′-androstane[3,2c]-pyrazole (17MM), referred to as compound **M** in the text, seems to selectively inhibit the process of 5α-reduction of testosterone in the epididymis.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Nossan, Correzzana, Milano, Italy) weighing between 300 and 350 g were used for this study. They were housed in an animal quarter, with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on 6.30 a.m.). Food and water were provided *ad libitum*.

After decapitation of the animals, the epididymes, immediately removed and stripped of fat, were separated into the caput, corpus and cauda regions. All operations were carried out at 4°C. Only the caput segment was used, because the 5α-reductase activity is higher in this region than in the other two segments [1], as recently confirmed also by the presence of the highest concentrations of mRNA found in this epididymal section [13]. In order to eliminate the connective tissue and isolate the epididymal tubules, the caput segment was preincubated for 45 min at 37°C in Krebs-Ringer phosphate buffer containing 0.5% of collagenase type II (Sigma Chemicals Co., St Louis, MO, U.S.A.). The tubules were then freed of the spermatozoa by pressing them out by hands. To ascertain the complete elimination of the spermatozoa from the tubule lumen, a light microscope examination was performed. After this, the tubules were minced and incubated. At autopsy the ventral prostates were also rapidly removed, dissected out and immediately minced; prostatic tissue was then incubated.

Incubation conditions

The minced rat caput epididymal and prostatic tissues (approx. 20 mg) were incubated in quadruplicates in 2 ml of Krebs-Ringer phosphate buffer (pH 7.0) containing 1 mM NADPH (Sigma) and [^{14}C]testosterone 6×10^{-7} M (sp. act. 200 μCi/mg, Amersham, England) previously purified. Each experiment was repeated five times. Vials without epididymal tissue provided the blank. The incubation was performed at 37°C for 2 h in a Dubnoff metabolic shaker under an atmosphere of 95% oxygen and 5% CO_2. This incubation time was selected because the reaction rate is linear and constant between 1 and 4 h. The reactions were stopped by freezing. Samples were kept at −20°C until extraction.

The different synthetic steroids to be evaluated (4-aza-3-oxo-steroids, androstene and androstane derivatives, see Figs 1 and 2 for their chemical structures) were added to the incubation media, dissolved in 20 μl of ethanol, at the concentrations of 10^{-7}, 10^{-6} and 10^{-5} M. The steroids 4-MA and 4-OH-A were considered as reference controls and used at the same molar concentrations as the molecules to be tested. The control vials contained only the same volume of ethanol without the addition of any compound. 4-MA was kindly provided by Dr G. H. Rasmusson (Merk, Sharp and Dohme, Research Laboratories, Rahway, NJ, U.S.A.). 4-OH-A was obtained from Dr A. M. H. Brodie (University of Maryland, Baltimore, U.S.A.), while the other steroids were supplied by Dr T. J. Lobl (Upjohn, Kalamazoo, MI, U.S.A.). The authors express their thanks for all these generous gifts.

Extraction and identification of the metabolites

Before extraction, known amounts of ^{3}H DHT were added to each vial in order to calculate the recoveries. The metabolites formed were extracted from the incubation media with 2 vol of 10 ml of diethyl-ether. The extracts were evaporated to dryness under a stream of nitrogen. The ether extracts were then dissolved in 100 μl of ethanol and applied on silica gel thin-layer chromatographic plates (60 F250, size 20 × 20 cm, thickness 0.25 mm; MERCK, Germany). A solution containing cold DHT and testosterone was added as a reference standard on each plate. The plates were developed in a solvent system of dichloromethane–diethylether (11:1, v/v) twice at 4°C. DHT was subsequently identified by exposing the plate to iodine vapour. The radioactivity present in each sample was measured using a Packard liquid scintillation spectrometer, model TRI-Carb 300°C.

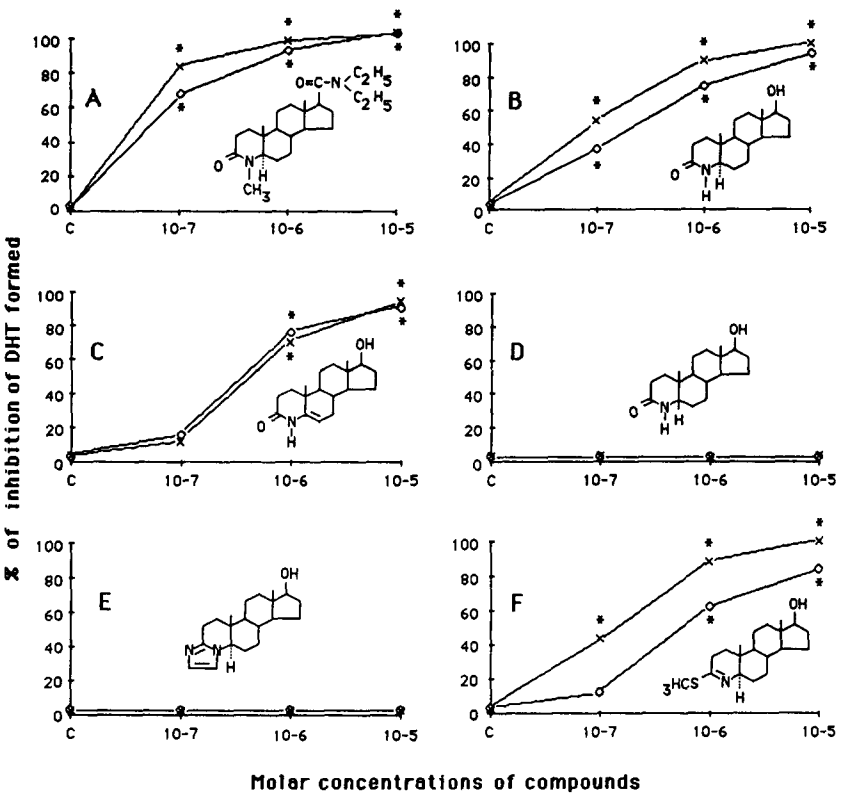

Fig. 1. *In vitro* effect of different steroid derivatives on the formation of DHT in the epididymis (◇) and ventral prostate (×) of the rat. Results are expressed as percent of inhibition of DHT formed vs control. *$2P \leqslant 0.05$ vs control.

Calculation of the results and statistical analysis

The 5α-reductase activity, expressed as pg of DHT formed in 2 h by mg of tissue, is referred to in the figures as the percent of inhibition of DHT formed vs controls. The data obtained were analysed by one-way analysis of variance. To determine the levels of significance of the experimental results vs the control the *t*-values were compared with the values of Dunnett's table for multiple comparisons [14]. The IC_{50} for each compound has been calculated by means of a specific programm (ALLFIT) developed by De Lean *et al.* [15] utilizing a Macintosh computer.

RESULTS

The results of the five experiments assessing the "*in vitro*" potency of 4-aza-3-oxo-steroids and of androstene and androstane derivatives as possible 5α-reductase inhibitors at the level of the epididymis are given respectively in Figs 1 and 2. 4-MA (compound **A**) has been arbitrarily selected as the reference molecule for the 4-aza derivatives and 4-OH-A (compound **G**) for the Δ4-3keto and androstane derivatives. The data are expressed as percent of inhibition of the amounts of DHT formed. As expected, 4-MA (compound **A**) is a potent 5α-reductase inhibitor, which develops a significant inhibition in the formation of DHT of 100, 90 and 65%, respectively at molar concentrations of 10^{-5}, 10^{-6} and 10^{-7} (Fig. 1). A dose related inhibition is also obtained with compound **B**; this steroid induces an inhibition of 90, 71 and 33%, respectively at molar concentrations of 10^{-5}, 10^{-6} and 10^{-7}. Compound **C** shows a different pattern of inhibition, in that it appears less effective than compound **B** at the lowest concentration considered (its percentage of inhibition is only 10% at 10^{-7} M), while the inhibition is identical to that of compound **B** at 10^{-6} and 10^{-5} M. Compound **D**, an isomer of **B**, as well as compound **E** are totally inactive. Compound **F** shows a percent of inhibition of 81, 62 and 10%, respectively at molar concentrations of 10^{-5}, 10^{-6} and 10^{-7}. When these steroids are incubated with rat prostate tissue, they show a pattern of 5α-reductase inhibition similar to that observed in the epididymis: compounds **B**, **C** and **F** are also inhibitory at the prostatic level, while compounds **D** and **E** are inactive (Fig. 1).

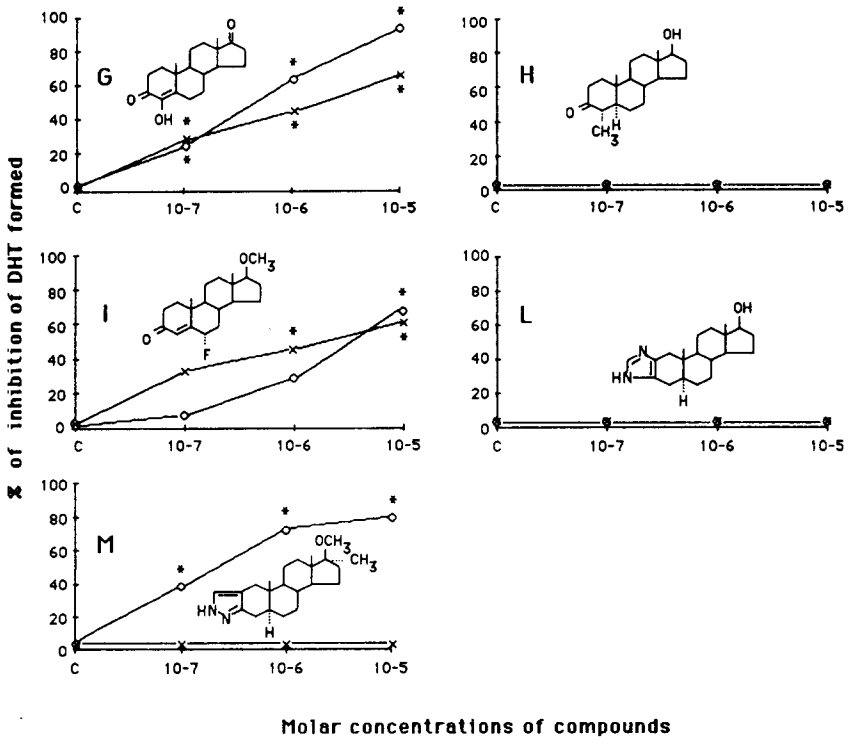

Fig. 2. *In vitro* effect of different steroid derivatives on the formation of DHT in the epididymis (◇) and ventral prostate (×) of the rat. Results are expressed as percent of inhibition of DHT formed vs control. *$2P \leqslant 0.05$ vs control.

The capability of the androstene and androstane derivatives to inhibit the transformation of testosterone to DHT is illustrated in Fig. 2. Compound **G**, used as a reference standard, shows a high inhibitory activity on the 5α-reductase in the epididymal tissue; its percent of inhibition corresponds to 90, 60 and 20% at the molar concentrations of 10^{-5}, 10^{-6} and 10^{-7}. Of the compounds of this series studied, only those classified as **I** and **M** were shown to be active. The percent of inhibition, at the concentrations used, are respectively 68, 27 and 5% for compound **I**, and 80, 68 and 34% for compound **M**. Compounds **H** and **L** are ineffective.

Compound **I**, at molar concentrations of 10^{-5}, 10^{-6} and 10^{-7}, shows an inhibiting effect on the 5α-reductase of the prostate of the same magnitude as that observed for the epididymal tissue. On the contrary, compound **M** is devoid of any inhibitory property on the prostatic 5α-reductase activity (Fig. 2).

Figure 3 shows the IC_{50}s of the compounds tested on the epididymal 5α-reductase and shows that compounds **B**, **G** and **M** have a similar potency and are more potent than the molecules **C**, **F** and **I**.

DISCUSSION

The present data show that a number of synthetic steroids are able to block the 5α-reductase activity of the caput of the epididymis, as shown by the diminished formation of DHT. In order to prove that this effect is due to the inhibition of the 5α-reductase directly and not to an enhanced activity of the enzyme converting DHT into 3α-diol, the formation of the diols was evaluated using labelled DHT as the substrate. No modification in the amounts of the

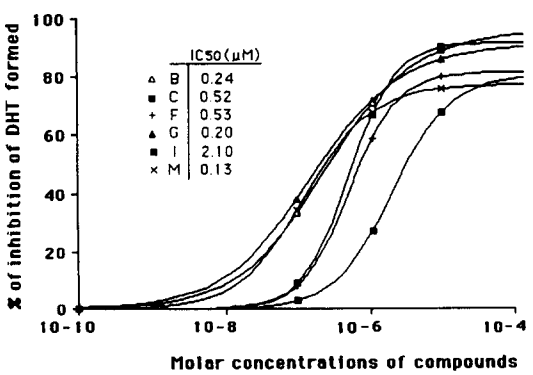

Fig. 3. Comparison of IC_{50}s of the different compounds tested on the epididymal 5α-reductase.

diols formed was observed in the presence of all the active inhibitors tested (data not shown).

Structural modifications of the A- and D-rings greatly influence the amounts of DHT formed with different degrees of magnitude. In particular, modifications of the A-ring which include a 3-oxo group, a 4-methyl-4-aza moiety and the (5α)-*trans* A-B ring junction (compound **A**) lead to maximal inhibitory activity on the formation of DHT. Actually, 4-MA (a known inhibitor of the prostatic 5α-reductase, as already mentioned) [11, 12] proved to be the most potent inhibitor of 5α-reductase in the epididymal tissue. Similar data have been reported by Cooke and Robaire [16]. Replacement of the 4-methyl group by a hydrogen (compound **B**) and the simultaneous incorporation of either a 5-6 double bond (compound **C**) or a 3-methylthio group (compound **F**) preserves the 5α-reductase inhibiting property of the compounds at least at the higher doses; on the contrary, the introduction of other functional groups renders the compounds practically inactive (see compounds **D** and **E**). It is interesting to underline that those compounds (i.e. **A**, **B**, **C** and **F**) which are effective on the 5α-reductase of the epididymis are also active on the enzyme of the prostate, while those compounds, which, like **D** and **E**, do not show any inhibiting property on the 5α-reductase of the epididymis are also inactive on the enzyme of the prostate.

The class of steroids possessing a Δ4-3-keto configuration shows changes in their 5α-reductase inhibiting property, depending on the different chemical modifications introduced in the molecules. 4-OH-A (compound **G**) produces a decrease in the formation of DHT at all concentrations tested. 4-OH-A has already been demonstrated in the authors' laboratory to be a good inhibitor of 5α-reductase both in the rat ventral prostate [5] and in human benign prostatic hyperplasia tissue [12]. Its inhibitory activity seems to be related to the presence of the Δ4-3-keto moiety in ring A, which appears to be a common feature of the competitive inhibitors of 5α-reductase [17, 18]. This observation is supported by the present data, since the 5α-reduced compounds **H** and **L**, which do not possess this configuration, lose their inhibiting activity. Compound **I**, which has a Δ4-3keto conformation, and which also possesses a fluoride atom at C-6 in the α position and a methyl-ether group in position C-17, shows some degree of inhibitory capacity, but its effect is significantly different from control values only at the higher concentrations. As in the case of the previous class of steroids, compounds **G** and **I**, which are active at the epididymal level are also good inhibitors of the 5α-reductase of the prostate; on the contrary compounds **H** and **L**, which are ineffective on the epididymal 5α-reductase, are also devoid of any effect on the prostatic enzyme.

The most interesting molecule among all those tested in this study appears to be compound **M** (17MM, or 17β-methoxy-17-methyl-(5α)-1H′-androstane[3,2c]pyrazole) which shows a remarkable and significant inhibiting effect on the 5α-reductase of the epididymis, but it is apparently devoid of any activity on the 5α-reductase present in the prostate. A significant and dose related decrease in the formation of DHT occurs in the epididymis, while in the prostate this steroid does not induce any modification in the amounts of DHT formed at all doses considered. The dichotomy of effects observed in the epididymis and in the prostate suggests that it may be possible to selectively interfere with the 5α-reductase of the epididymis, without affecting the enzyme present in the prostate. To the authors' knowledge no report on the differential activity of this or analogous compounds has ever been presented.

At present, it is difficult to explain the reasons for this selectivity of action. Several hypotheses may be put forward. The first possibility would be a facilitated transport of compound **M** in the epididymal cells. It is interesting to recall that this steroid possesses a very low affinity for androgen receptors (0.006 as compared to that of DHT), but binds to ABP (androgen binding protein) [19]. It is known that ABP, a secretory product of the Sertoli cell, facilitates transport of androgens from the seminiferous tubules to the epididymis and possibly from the interstitial fluid to epididymal cells. Consequently, one might postulate that compound **M** easily reaches epididymal cells because of its facilitated transport via ABP, which has recently been shown to be internalized in the epididymal cells [20]. A second possibility would be the presence of two different 5α-reductases, one in the epididymis and the other in the prostate. Martini *et al.* [21] and Rennie *et al.* [22] have postulated the existence of different 5α-reductases, respectively in the normal rat prostate and in human benign hyperplasia. This result has recently been confirmed by the isolation of two different cDNA coding for two different 5α-reductases (designated 1 and 2, respectively) [23]. It is interesting that

Andersson *et al.* [23] have found that only 5α-reductase 2 is inhibited by finesteride, a new 5α-reductase inhibitor.

Work is presently in progress in the authors' laboratory to verify whether the suggested selectivity of action of compound **M** on the 5α-reductase of the epididymis observed "*in vitro*" is also maintained when the compound is given "*in vivo*". Were this hypothesis correct, compound **M** should be a useful research tool in differentiating the action of different androgens in different organs. It is also possible that its ability to bring about a reduction in the formation of DHT in the epididymis, but not in the prostate, might be exploited in the clinical practice. The possibility of interfering selectively with the formation of DHT at the level of the epididymis, coupled with the fact that DHT is necessary for the maturation of the spermatozoa (see Introduction) suggests a new way to regulate male fertility, obviously if compound **M** does not show toxicity or other side effects.

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