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Androgen binding profiles of two distinct nuclear androgen receptors in Atlantic croaker (*Micropogonias undulatus*)

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Abstract

In the present study, the binding affinities of 28 androgens for two nuclear androgen receptors (AR), termed AR1 and AR2, in Atlantic croaker (*Micropogonias undulatus*) brain and ovarian tissues, respectively, were determined using competitive binding assays. The 5α-reduction of steroids, in general, increased the metabolite's binding affinity for AR2 while decreasing it for AR1. In addition, few androgens bound to AR1 with high affinity and modifications to the basic 3-ketone,4-ene,17β-hydroxy structure of testosterone usually reduced its binding affinity for AR1. However, androgens with ketone groups at the 3- and 17-position bound with high affinity to AR1 provided that the androgen had either a 5α-reduced A-ring or a third ketone group at the 11-position. This suggests that there may be several high affinity conformations that AR1 can occupy depending upon whether an androgen possesses a ketone or a hydroxyl group at the 17-position. The binding of androgens to AR2 showed a more predictable pattern, 5α-reduced steroids bound better than 4-ene steroids and any changes to the basic 3-keto,17-hydroxy motif of 5α-dihydrotestosterone lowered the binding affinity of a steroid. However, these structural changes often caused only minor decreases in binding affinity, such that AR2 has a broader affinity for androgens and a greater affinity than AR1 for structurally diverse androgens. Widely different androgen binding affinities of AR1 and AR2 suggest that these two nuclear androgen receptors may mediate the physiological actions of different androgens in teleosts.

Keywords: Androgen; Androgen receptor; Fish; Brain; Ovary

1. Introduction

Two distinct nuclear androgen receptors (AR), termed AR1 and AR2, have been previously biochemically characterized in the teleost fish, Atlantic croaker *Micropogonias undulatus* [1]. Moreover, AR1 was found only in brain tissue, whereas AR2 was found in both gonadal and brain tissues. The two ARs have different physical characteristics as well as different steroid binding specificities, based upon a limited number of steroids tested. While the physiological functions and the specific cellular and tissue distributions of these two ARs are not currently known, their different characteristics suggest that they mediate the actions of different androgens. Multiple forms of nuclear steroid receptors have been found across vertebrate lines. Multiple subtypes of the estrogen receptor (ER) have been identified in mammals [2], birds [3,4], and fish [5,6], and multiple isoforms of the AR are present in amphibians [7] and mammals [8]. This extensive multiplicity, found within the nuclear steroid receptor superfamily, suggests that the alternate forms of these receptors are an important component mediating the pleiotropic responses of steroid hormones.

High levels of androgens are present in the blood of both male and female teleosts during gonadal recrudes-

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cence. Testosterone (T) is the predominant androgen in the circulation of most female fish. However, in addition to T, 11-oxygenated androgens are commonly found at high concentrations in the plasma and tissues of male fish and are generally considered to be the most important physiological androgens in males (reviewed by [9–11]). The major 11-oxygenated androgens in teleosts, 11β-hydroxytestosterone (11β-HT), 11-ketoandrostenedione (11-KA) and 11-ketotestosterone (11-KT) are synthesized via the steroidogenic enzymes 11β-hydroxylase and 11β-hydroxy steroid dehydrogenase [12]. The study of androgen function in teleosts has previously focused upon the paradigm of androgens acting through a single AR. However, the discovery of two ARs in fish with different binding affinities and tissue distributions suggests that different androgens may mediate the broad spectrum of androgen responses in different target tissues during gonadal differentiation [13], spermatogenesis [14] and the induction of secondary sexual characteristics [15–17]. However, there have been no detailed comparisons of the suite of androgens present in various target tissues and their relative binding affinities for the AR subtype or isoform which would indicate which androgens are primary mediators of the androgenic responses.

It was previously shown that croaker AR1 is specific for androgens since it has no affinity for estradiol or progestogens. However, of the seven androgens examined only T bound with high affinity whereas 5α-dihydrotestosterone (5α-DHT) and 11-KT had low affinity [1]. A similar androgen binding profile of the AR1 receptor was observed in the brain tissue of kelp bass (*Paralabrax clathratus*) [18]. In addition, the ARs described in brown trout (*Salmo trutta*) skin [19], goldfish (*Carassius auratus*) brain tissue [20] and rainbow trout (*Oncorhynchus mykiss*) lymphocytes [21], also possess specific, high affinity for T and low affinity for DHT and 11-KT. The similarities in binding affinities of these ARs suggest that AR1 is found throughout teleosts, and is heretofore unique to this vertebrate group.

The binding profile of croaker AR2 is distinct from that of AR1, with DHT, T and 11-KT, as well as the synthetic androgens 17β-hydroxy-17α-methyl-5α-androstan-3-one (MDHT),17β-hydroxy-17α-methyl-4-androsten-3-one (MT) and 17β-hydroxy-7α,17α-dimethyl-4-estren-3-one (mibolerone) all bind with high affinity to AR2 [1]. Similar androgen affinity profiles were found with the kelp bass AR2 [18] as well as with the AR detected in coho salmon (*Oncorhynchus kisutch*) ovaries [22]. Recently, cDNA sequences for two AR subtypes ARα and ARβ [23] from Japanese eel (*Anguilla japonica*) testes where, in vitro, DHT, 11-KT and T all induced high transcriptional activity, indicating that these receptors may have binding characteristics similar to AR2. These data suggest that AR2 is likely to be present throughout teleosts. Moreover, AR2 has biochemical and steroid binding characteristics similar to those of mammalian ARs [24].

In the present study, the binding affinities of a broad range of androgens for AR1 and AR2 in Atlantic croaker brain and ovarian tissues were examined in order to, more precisely, delineate the structural characteristics necessary for high affinity binding to the two ARs. Androgens with 11-oxygenated substitutions and various other substitutions at the 3- and 17-positions, as well as androgens with differing degrees of saturation were examined in order to gain insight into the specific requirements of the AR1 and AR2 binding sites. In addition, the binding affinities for the two ARs of various synthetic androgens with different methyl-group substitutions and the affinities of the steroidal antiandrogen cyproterone acetate and the nonsteroidal antiandrogens RU 58841, RU 56187 and RU 3786 were determined.

2. Materials and methods

2.1. Chemicals

[1,2,6,7-^{3}H]Testosterone (92.0 Ci/mmol), [1,2,4,5,6,7-^{3}H] 5α-dihydrotestosterone (127.0 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and stored at −20°C. The unlabeled steroids were purchased from either Steraloids, (Wilton, NH) or from Sigma (St. Louis, MO). Cyproterone acetate and flutamide were purchased from Sigma and R1881 from New England Nuclear. RU 3786, RU 58841 and RU 56187 were gifts from Roussel Uclaf (Cedex, France) and Mibolerone was a gift from the Upjohn Laboratories (Kalamazoo, MI). All steroids and non-steroidal antiandrogens were stored in 95% ethanol at −20°C. Chemicals and salts used for making the buffers were purchased from Sigma and Fisher Scientific (Pittsburgh, PA). The scintillation cocktail was a mixture of 4 l toluene, 16 g PPO (7,5-diphenyloxazole) and 0.4 g POPOP (1,4-bis[5-phenyl-2-oxazolyl]-benzene) and 400 ml methanol.

2.2. Animals and tissue sampling

Adult male and female Atlantic croaker, between 14–28 cm in length, were collected during the reproductive season in the fall by either gill net or trawl from the bays near Port Aransas, TX. Fish were maintained in circular, recirculating tanks under constant photoperiod and temperature regimes and fed a commercial fish food diet daily. Fish were acclimated in the laboratory for at least 1 month prior to any tissue collection. Fish were rapidly decapitated and tissues were removed, placed on ice and used immediately or

frozen on dry-ice and stored at −80°C for up to 3 months.

2.3. Buffers

Assay buffers for AR1 measurement were H-1, the homogenization buffer (50 mM Tris–HCl, 1 mM EDTA, 12 mM monothioglycerol, 30% glycerol (v/v), pH 7.5 at 4°C); W, the washing buffer (10 mM Tris–HCl, 2 mM $MgCl_2$, 2 mM monothioglycerol, 250 mM sucrose, 10% glycerol (v/v), pH 7.5 at 4°C) and E-1, the extraction buffer (H-1 + 0.7 M KCl). The assay buffer for AR2 measurement was H-2, the homogenization buffer (50 mM Tris–HCl, 10 mM sodium molybdate, 1 mM EDTA, 12 mM monothioglycerol, 10% glycerol (v/v), pH 7.4 at 4°C).

2.4. Preparation of tissue for AR1 and AR2 assays

Whole brain tissue, comprising the olfactory bulbs and everything posterior to the medulla oblongata including the pituitary, were prepared as described previously [1]. Briefly, brain tissue homogenates in H-1 buffer (1:10 w/v) were centrifuged at $2500 \times g$ for 15 min and the resulting supernatant (S1) was spun at $160{,}000 \times g$ for 1 h. The cytosolic fraction (S1) was charcoal-stripped to remove endogenous steroids. Ovarian tissue homogenates were prepared in H-2 buffer (1:10 w/v) in an identical manner to that for preparing brain cytosolic fractions. The initial $2500 \times g$ pellet from the brain tissue homogenates was washed three times in ice-cold W buffer. Following the wash, the pellet was resuspended in E-1 buffer (1:10 initial tissue w/v) and incubated for 1 h with vortexing at 15 min intervals. The suspension was then centrifuged at $160{,}000 \times g$ for 1 h and the resultant supernatant was the nuclear fraction. The tissue preparations were either used immediately or frozen at −80°C where binding activity remained constant for at least 1 week.

2.5. Competition studies with steroids

The competitive binding of various unlabeled steroid and non-steroidal competitors was determined by pipetting; first, unlabeled competitor, dissolved in 95% ethanol, into test tubes and allowing it to completely dry, second, 50 μl of [^{3}H]ligand in buffer and third, 250 μl of tissue sample. For the determination of binding to AR1 4 nM [^{3}H]T in buffer E-1 was used, while 1 nM [^{3}H]DHT in buffer H-2 was used for AR2. Samples were vortexed and incubated for 18–22 h for AR1 as well as for AR2. Competition curves of unlabeled T and DHT were used as standards for each of the AR1 and AR2 assays, respectively. The relative binding affinities (RBAs) of steroids for AR1 and AR2 were expressed as a percentage of the maximum specific binding of T and DHT, respectively.

2.6. Separation of bound from free steroid

Free steroid was separated from bound by the dextran-coated charcoal (DCC) method. An equal volume of DCC (50 mM Tris–HCl, 1 mM EDTA, 10% glycerol (v/v), 1% Norit-A charcoal (w/v), 0.1% dextran T-70 (w/v), pH 7.5 at 4°C) was added to the samples and the mixture was incubated for 5 min prior to centrifugation at $3000 \times g$ for 5 min at 4°C. The supernatants were decanted into 7 ml scintillation vials, 5 ml of the standard scintillation cocktail was added and the radiation was measured. The radioactivity within each sample was determined by counting for 5 min in a Beckman LS 6000SC scintillation counter (Beckman Instruments, Fullerton, CA).

3. Results

The binding curves presented in Figs. 1–6 were parallel indicating that the binding was competitive between the various unlabelled competitors and [^{3}H]T for AR1 and [^{3}H]DHT for AR2, allowing the EC_{50}s to be determined and the RBAs to be calculated (defined in Table 1, see also for key to steroid abbreviations). Parallel binding was not observed for the steroids 11-KA and 11-KDHA, which demonstrated biphasic binding curves to AR2 (Fig. 4).

3.1. Influence of double bonds within the A- and B-rings

Compared to T which has a 4–5 double bond, the steroid 17β-hydroxy-1(5α)-androsten-3-one (Δ1-DHT), which has a 1–2 double bond, bound with 500-times less affinity for AR1 (see Table 1, Fig. 1). This is in contrast to 17β-hydroxy-1,4-androstadiene-3-one (Δ1-T) with both 1–2 and 4–5 double bonds, which bound with an RBA of 114%, whereas DHT, with no double bond within the A-ring, had an RBA of only 8%. The addition of a 6–7 double bond to the B-ring of T, 17β-hydroxy-4,6-androstadiene-3-one, decreased the RBA to 16.7%.

The presence of double bonds within the A- and B-rings also influences the affinity of the steroids for AR2, however, the position of the double bond is not as critical (see Table 1, Fig. 1). Compared to 5α-DHT, the addition of a single double bond to the A-ring at either the 1–2 position, 17β-hydroxy-1(5α)-androsten-3-one, or the 4–5 position, T, causes a similar reduction in the RBAs to 57% and 50%, respectively. The 17β-hydroxy-1,4-androstadiene-3-one with multiple double bonds within the A-ring has an RBA of

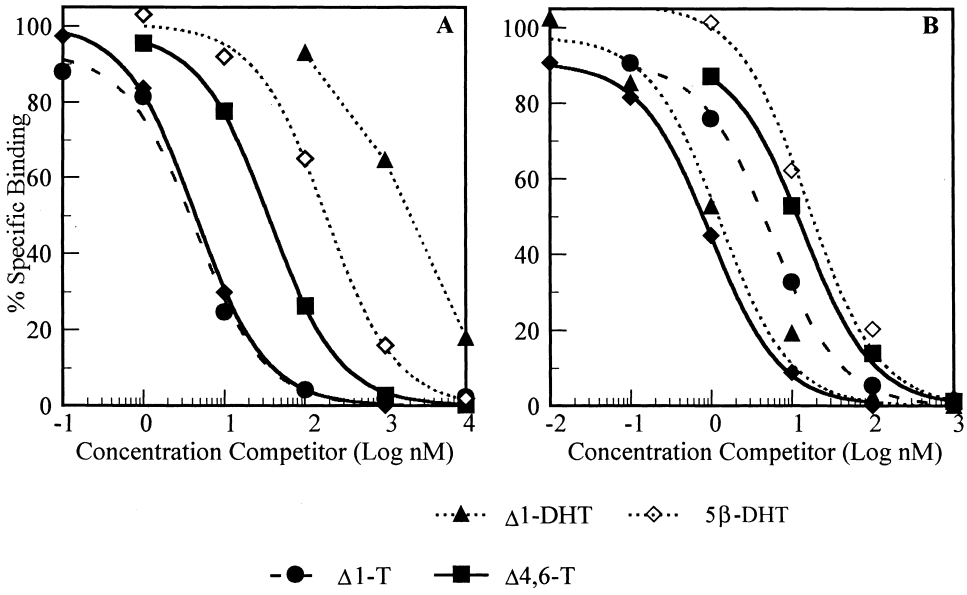

Fig. 1. Competition curves of the binding of androgens with varying degrees of saturation within the A- and B-rings to (A) AR1 in brain nuclear extracts relative to T (●) and to (B) AR2 in ovarian cytosolic extracts relative to DHT (◆). Each data point is the average of 2–4 assays with SEM < 5–10%. See Table 1 for key to steroid abbreviations.

17.8% and 17β-hydroxy-4,6-androstadiene-3-one, with a double bond within both the A- and B-rings has an RBA of 6.7%. Both AR1 and AR2 demonstrate stereospecificity for five reduced androgens since, compared to 5α-DHT, 5β-DHT bound with four-times lower affinity to AR1 and bound with 25-times lower affinity to AR2 (Table 1).

3.2. Influence of ketone and hydroxyl groups at the 3- and 17- positions

The C19 steroid 5α-androstane demonstrated no affinity for AR1, however 5α-androstan-17β-ol had an RBA of 0.08% and 5α-androstan-3-one had an RBA of 0.5% (see Table 1, Fig. 2). Hydroxylation of the 3-

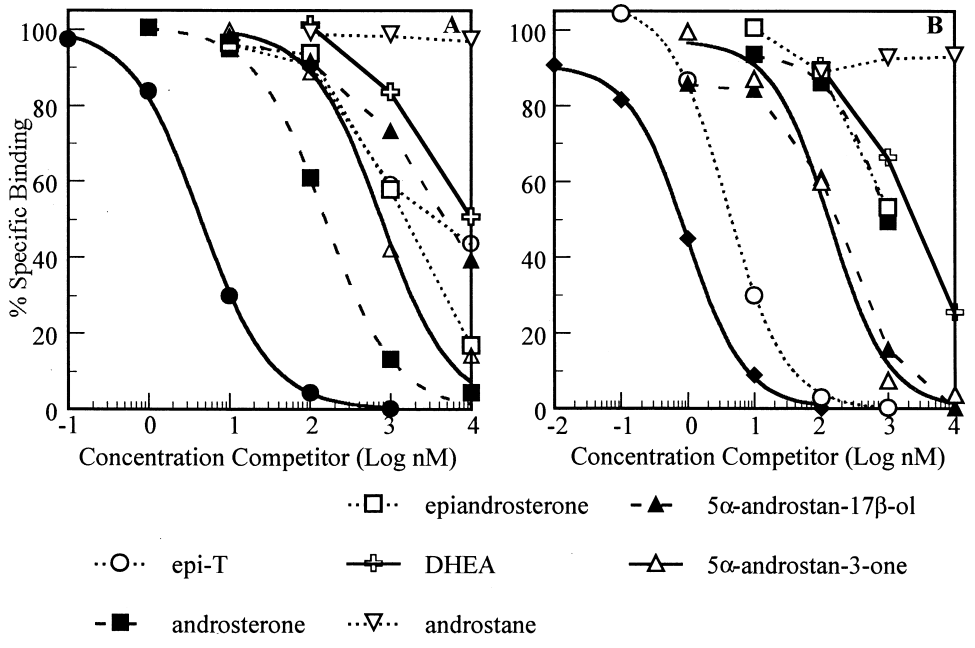

Fig. 2. Competition curves of the binding of androgens with substitutions at the 3- and 17-positions to (A) AR1 in brain nuclear extracts relative to T (●) and to (B) AR2 in ovarian cytosolic extracts relative to DHT (◆). Each data point is the average of 2–4 assays with SEM < 5–10%. See Table 1 for key to steroid abbreviations.

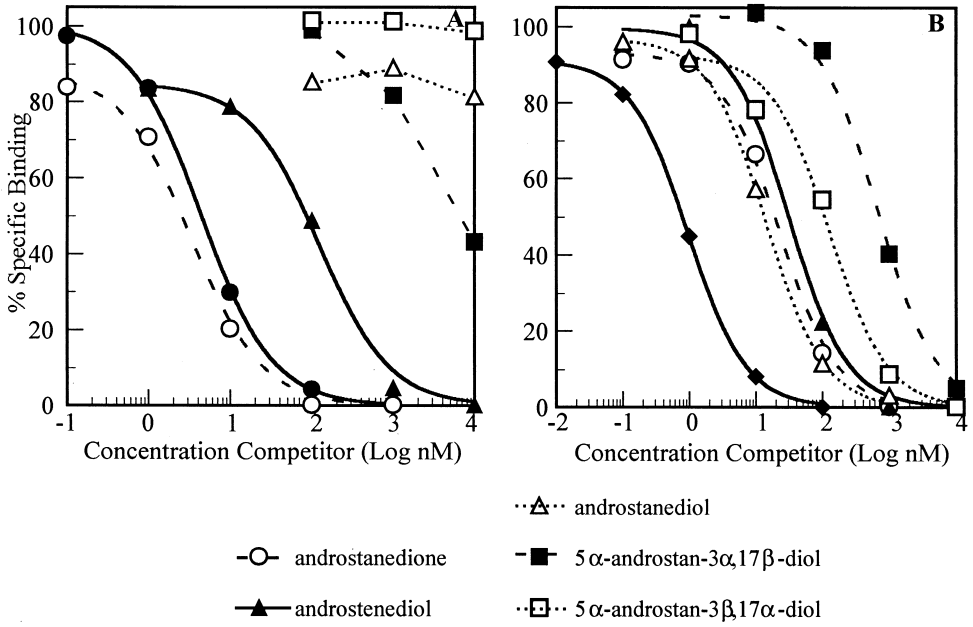

Fig. 3. Competition curves of the binding of androstane-3,17-diols and androstane-3,17-diones to (A) AR1 in brain nuclear extracts relative to T (●) and to (B) AR2 in ovarian cytosolic extracts relative to DHT (◆). Each data point is the average of 2–4 assays with SEM < 5–10%. See Table 1 for key to steroid abbreviations.

ketone of T to 4-androstene-3β,17β-diol lowers its RBA for AR1 to 4.4% (Fig. 3). The additional 5α-reduction of 4-androstene-3β,17β-diol to 5α-androstane-3β,17β-diol eliminated the affinity of this steroid for AR1 at concentrations up to 10,000 nM (Fig. 3). The 3α-epimer 5α-androstane-3α,17β-diol demonstrates slightly higher affinity (RBA of 0.07%) for AR1 suggesting stereospecificity at the 3-position. Likewise, androsterone with a 3α-hydroxyl group has a higher affinity (RBA of 2.7%) than epiandrosterone with a 3β-hydroxyl group (RBA of 0.25%) (Fig. 2). The addition of a 4–5 double bond to androsterone forming dehydroepiandrosterone (DHEA) reduces its RBA for AR1 to 0.01%. AR1 is also stereospecific at the 17-

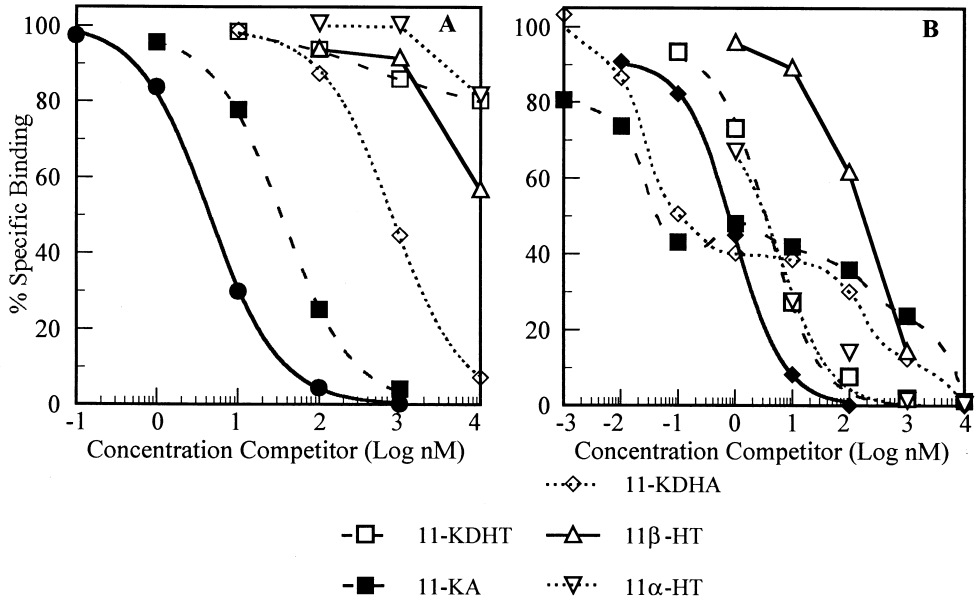

Fig. 4. Competition curves of the binding of 11-oxygenated androgens to (A) AR1 in brain nuclear extracts relative to T (●) and to (B) AR2 in ovarian cytosolic extracts. Each data point is the average of 2–4 assays with SEM < 5–10%. See Table 1 for key to steroid abbreviations.

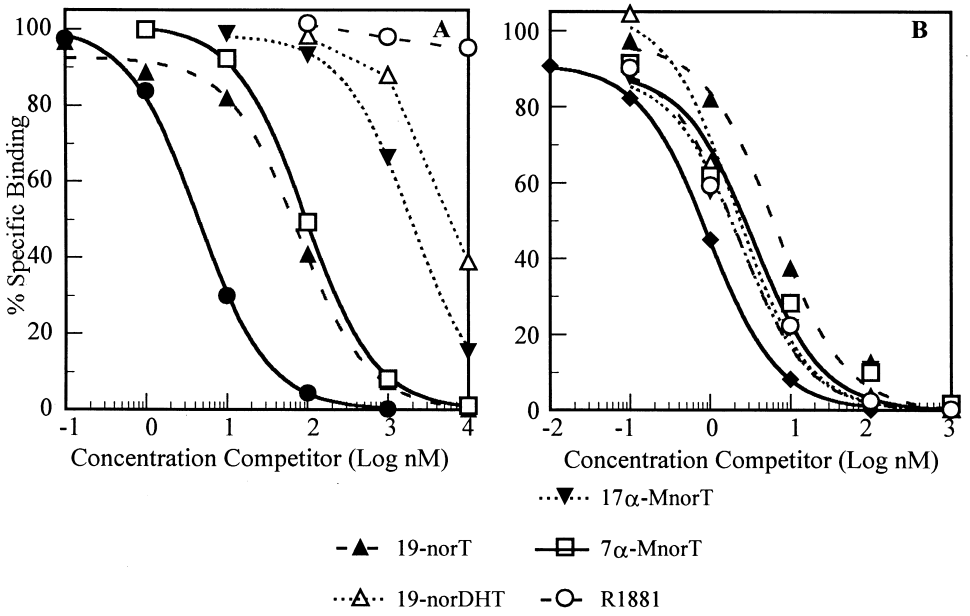

Fig. 5. Competition curves of the binding of androgens with varying degrees of methylation to (A) AR1 in brain nuclear extracts relative to T (●) and to (B) AR2 in ovarian cytosolic extracts relative to DHT (◆). Each data point is the average of 2–4 assays with SEM < 5–10%. See Table 1 for key to steroid abbreviations.

position since epitestosterone, 17α-hydroxy-4-androsten-3-one, has an RBA of 0.13%. The substitution of a 17-keto group for the 17β-hydroxyl group of DHT, forming 5α-androstane-3,17-dione, bound to AR1 with an RBA of 153% (Fig. 3).

AR2 had no affinity for 5α-androstane. 5α-androstan-3-one and 5α-androstan-17β-ol bound to AR2 with similar RBAs of 0.5% and 0.73%, respectively (see Table 1, Fig. 2). Hydroxylation of the 3-ketone of DHT to 5α-androstane-3β,17β-diol lowers its RBA for AR2 to 6.7% (Fig. 3). The affinity of AR2 for the 3,17-diols is stereospecific with the 5α-3β,17β-diol binding with the highest affinity, whereas the 3β,17α-diol, (5α-androstane-3β,17α-diol), had an RBA of 0.53% and the 3α,17β-diol, (5α-androstane-3α,17β-diol), bound with an RBA of 0.19%. The addition of

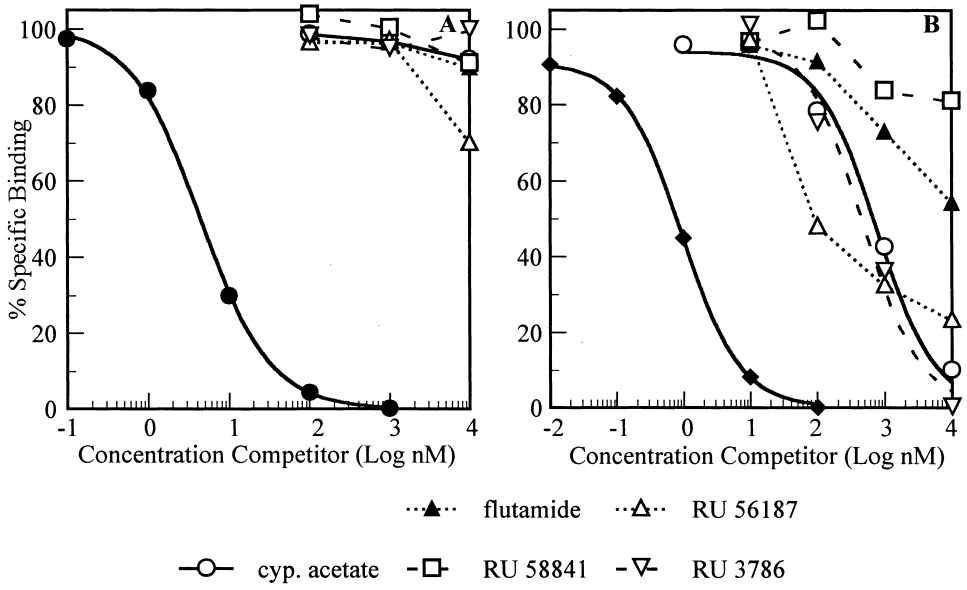

Fig. 6. Competition curves of the binding of antiandrogens to (A) AR1 in brain nuclear extracts relative to T (●) and to (B) AR2 in ovarian cytosolic extracts relative to DHT (◆). Each data point is the average of 2–4 assays with SEM < 5–10%. See Table 1 for key to steroid abbreviations.

a 4–5 double bond to the 3β,17β-diol forming 4-androstene-3β,17β-diol lowers the affinity for AR2 to 2.7%. The substitution of a 17-keto group for the 17β-hydroxyl group of DHT, forming 5α-androstane-3,17-dione, lowers the RBA for AR2 to 4.2% (Fig. 3). Androsterone and its 3β-epimer bind with similar RBAs of 0.08% and 0.07% for AR2, respectively. DHEA, with a 4–5 double bond, has an RBA of 0.03%.

3.3. Influence of ketone and hydroxyl groups at the 11-position

The addition of an 11-oxygenated group to either T or DHT nearly eliminated their capability to bind to AR1 (see Table 1, Fig. 4). 17β-hydroxy-5α-androstane-3,11-dione and 11α,17β-dihydroxy-4-androsten-3-one demonstrated less than 20% displacement at 10,000 nM and 11β,17β-dihydroxy-4-androsten-3-one demon-

Table 1
Relative binding affinities of various steroids for brain nuclear AR1 and ovarian cytosolic AR2

Steroid (common name, abbreviation)	EC_{50} (nM)[a]		RBA (%)[b]	
	AR1	AR2	AR1	AR2
17β-hydroxy-4-androsten-3-one (testosterone, T)	4	1.6	100	50
17β-hydroxy-5α-androstan-3-one (dihydrotestosterone, DHT)	50	0.8	8	100
17β-hydroxy-5β-androstan-3-one (5β-dihydrotestosterone, 5β-DHT)	190	20	2.1	4
17α-hydroxy-4-androsten-3-one (epitestosterone, epiT)	3000	4.5	0.13	17.8
5α-androstane	nd[c]	nd	0	0
5α-androstan-3-one	200	160	0.5	0.5
5α-androstan-17β-ol	5000	110	0.08	0.7
3α-hydroxy-5α-androstan-17-one (androsterone)	150	1000	2.7	0.08
3β-hydroxy-5α-androstan-17-one (epiandrosterone)	1600	1100	0.25	0.07
3α-hydroxy-5-androsten-17-one (dehydroandrosterone, DHEA)	10,000	2600	0.01	0.03
3,17 Diones				
5α-androstane-3,17-dione (androstanedione)	2.6	19	153	4.2
3,17 Diols				
4-androstene-3β,17β-diol (androstenediol)	90	30	4.4	2.7
5α-androstane-3β,17β-diol (androstanediol)	nd	12	0	6.7
5α-androstane-3α,17β-diol	6000	440	0.07	0.2
5α-androstane-3β,17α-diol	nd	150	0	0.5
11-Oxygenated androgens				
17β-hydroxy-5α-androstane-3,11-dione (11-ketodihydrotestosterone, 11-KDHT)	nd	4	0	20
4-androstene-3,11,17-trione (androstenetrione, 11-KA)	30	0.05	13.3	2000
5α-androstane-3,11,17-trione (androstanetrione, 11-KDHA)	500	0.15	0.5	533
11β,17β-dihydroxy-4-androsten-3-one (11β-hydroxytestosterone, 11β-HT)	nd	200	0	0.4
11α,17β-dihydroxy-4-androsten-3-one (11α-hydroxytestosterone, 11α-HT)	nd	4	0	20
Androgens with multiple double bonds				
17β-hydroxy-1,4-androstadiene-3-one (Δ1-testosterone, Δ1-T)	3.5	4.5	114	17.8
17β-hydroxy-1(5α)-androsten-3-one (Δ1-dihydrotestosterone, Δ1-DHT)	2000	1.4	0.2	57
17β-hydroxy-4,6-androstadiene-3-one	24	12	16.7	6.7
Methylated and 19-nor androgens				
17β-hydroxy-4-estren-3-one (19-nortestosterone, 19-norT)	70	6	5.7	13.3
17β-hydroxy-5α-estran-3-one (19-nordihydrotestosterone, 19-norDHT)	7000	2.4	0.06	33.3
17β-hydroxy-17α-methyl-4-estren-3-one (17α-MnorT)	2000	2	0.2	40
17β-hydroxy-7α-methyl-4-estren-3-one (7α-MnorT)	100	2.8	4	28.6
17β-hydroxy-17α-methyl-4,9,11-estratriene-3-one (R1881)	nd	1.3	0	62
Steroidal and nonsteroidal antiandrogens				
Cyproterone acetate	nd	700	0	0.1
Flutamide	nd	nd	0	0
RU 58841	nd	nd	0	0
RU 56187	nd	80	0	1
RU 3786	nd	400	0	0.2

[a] EC_{50} of each compound is calculated from competition curves (see Figs. 1–6) and is equal to the concentration of competitor necessary to displace 50% of either 4 nM [^{3}H]T from AR1 or 1 nM [^{3}H]DHT from AR2.

[b] RBA is calculated from the ratio of the EC_{50}s of the various competitors to the EC_{50}s of either T or DHT. The RBAs of T and DHT were set at 100 for AR1 and AR2 binding, respectively.

[c] nd = no displacement of [^{3}H]ligand at the maximum concentration of competitor.

strated only 45% displacement at 10,000 nM. On the other hand, 4-androstene-3,11,17-trione bound with higher affinity to AR1 with an RBA of 13.3% and its 5α-reduced counterpart 5α-androstane-3,11,17-trione had an RBA for AR1 of 0.5%.

The addition of an 11-keto group to DHT forming 17β-hydroxy-5α-androstane-3,11-dione lowered its RBA for AR2 to 20% (see Table 1, Fig. 4). The 11β-hydroxylated steroid, 11β,17β-dihydroxy-4-androsten-3-one had a low affinity for AR2 (RBA of 0.4%), whereas the 11α-hydroxylated steroid 11α,17β-dihydroxy-4-androsten-3-one (RBA of 20%) had only five-fold lower affinity than DHT for AR2, thereby demonstrating stereospecific binding of 11-hydroxylated androgens. The binding curves for both 4-androstene-3,11,17-trione and 5α-androstane-3,11,17-trione were biphasic. At low concentrations, both steroids demonstrate high affinity binding with RBAs of 2000% and 533%, respectively, whereas at high concentrations both 11-KA and 11-KDHA bind with low affinity. Similar biphasic binding curves were obtained with 11-KA and 11-KDHA when no sodium molybdate was present in the buffer (data not shown).

3.4. Methylated and 19-nor androgens

The two androgen receptors showed marked differences in their capabilities to bind androgens without the 19-methyl group, 19-nor androgens or androgens with additional methyl groups (see Table 1, Fig. 5). 17β-hydroxy-4-estren-3-one had an RBA of 5.7% for AR1 whereas 17β-hydroxy-5α-estran-3-one had an RBA of only 0.06%. The addition of methyl groups at the 17α- and 7α-positions of 17β-hydroxy-4-estren-3-one produced RBAs for AR1 of 0.2% and 4%, respectively. The synthetic androgen R1881, with a 17α-methyl group demonstrated no affinity for AR1 at concentrations of 10,000 nM. On the other hand, R1881 has an RBA for AR2 of 61.5% followed by 17β-hydroxy-17α-methyl-4-estren-3-one with an RBA of 40%. The RBA of this steroid for AR1 was 0.2%. 17β-hydroxy-5α-estran-3-one with an RBA of 33.3% has a higher affinity for AR2 than 17β-hydroxy-4-estren-3-one with an RBA of 13.3%.

3.5. Steroidal and non-steroidal antiandrogens

AR1 had no affinity for either the steroidal anti androgen cyproterone acetate or the four non-steroidal antiandrogens flutamide, RU 58841, RU 3786 or RU 56187 (see Table 1, Fig. 6). RU 56187 did demonstrate 30% displacement of [^{3}H]T from AR1 at a concentration of 10,000 nM. In contrast, cyproterone acetate has an RBA for AR2 of 0.11% and RU 3786 and RU 56187 have RBAs of 0.2% and 1%, respectively. However, the binding curve of RU 56187 was not parallel to the DHT curve at high concentrations (1000 and 10,000 nM).

4. Discussion

The steroid specificity studies with Atlantic croaker ARs show that the 5α-reduction of steroids, in general, increases the metabolite's affinity for AR2 while decreases it for AR1. For example, the RBA for AR1 of T > DHT, androstenediol > androstanediol, 11-KA > 11-KDHA and 11-KT > 11-KDHT. The synthetic androgens also follow this trend for AR1 with the RBA of 19-norT > 19-norDHT and 17β-hydroxy-17α-methyl-4-androsten-3-one (MT) > 17β-hydroxy-17α-methyl-5α-androstan-3-one (MDHT) [1]. The degree of saturation and the placement of double bonds within the A-ring is critical for maximum binding affinity to AR1 for most androgens, since 17β-hydroxy-1,4-androstadien-3-one has an RBA similar to T, whereas 17β-hydroxy-1(5α)-androsten-3-one has an RBA of only 0.2%, despite having a 1–2 double bond within the A-ring. However, the exception to the paradigm of 4-ene steroids demonstrating higher affinity for AR1 are androstenedione and androstanedione which have RBAs for AR1 of 0% [1] and 153%, respectively. On the other hand, AR2 has a greater affinity for 5α-reduced metabolites than the corresponding 4-ene steroids. All of the 5α-reduced steroids tested within this study as well as those examined previously [1] bound with higher affinity to AR2 than the corresponding 4-ene steroid. For example, the RBA of DHT > T, 5α-androstane-3β,17β-diol > 4-androstene-3β,17β-diol and 11-KDHA > 11-KT [1]. However, certain 4-ene steroids, such as T, do still demonstrate high affinity for AR2.

These binding data suggest that the steroidogenic enzyme 5α-reductase may play a role in determining which AR is activated in teleosts. In mammals, 5α-reductase has an integral role in amplifying the androgenic signal in a tissue specific manner by converting T to the more active androgen DHT [25]. It is possible that 5α-reductase could act similarly in fish, although the cell- and tissue-specific distributions and activities of both ARs and 5α-reductase have not been determined. 5α-reductase activity has been found within brain tissue of male and female goldfish, *Carassius auratus*, and toadfish *Opsanus tau* [26] and in the testes of the urohaze-goby, *Glossogobius olivaceus*, [27] and 5α-reduced metabolites are frequently found in gonadal and peripheral tissues of both male and female teleosts [10]. The 5α-reductase is clearly important within the neuroendocrine system in goldfish, the only teleost where it has been studied, with enzyme levels correlating with the seasonal reproductive cycle and with levels comparable to those found in brain tissue of other ver-

tebrates [26,28]. However, the importance of 5α-reduced androgens, such as DHT, in teleosts has not been thoroughly examined. While 5α-reduced metabolites have been measured throughout teleosts, their presence has not been correlated with a specific physiological role, thus it is difficult to determine whether the high affinity binding of DHT or androstanedione have physiological relevance. Latz and Reinboth [29], based upon their analysis of androgen metabolism in skin of rainbow trout, suggest that 5α-reduction may be an important component of the androgen signaling process. However, the importance of 5α-reduced steroids is likely to vary amongst fish. For example, in certain teleost species it appears that 5α-reductase is more active in females than in males and in Australian lungfish (*Neoceratodus forsteri*) 5α-reductase activity may be absent [30]. To the best of our knowledge, androstanedione has not been identified in teleosts, however androstenedione is commonly measured within teleosts [9–11] and given the abundance of 5α-reductase it is conceivable that androstanedione could be produced within teleosts. As a result of its high affinity towards AR1 it would be interesting to examine whether this steroid has any biological activity or relevance in the Atlantic croaker.

The presence of a 3-keto group is necessary for high affinity binding of androgens to both AR1 and AR2. Likewise, the 17-position has a strong influence on binding affinity. No steroid bound with high affinity, defined as an RBA greater than 10%, to AR2 without either a 17β- or 17α-hydroxyl group. Similarly, the presence of a 17β-group is important for high affinity binding to AR1, the presence of a 17-ketone group can also produce high affinity binding provided the androgen is either 5α-reduced, i.e. androstanedione, or has an 11-keto group, i.e. 11-KA. The important contribution that the 3-keto position makes upon the specificity of steroids for AR1 is supported by the findings that the addition of a 3-keto group to 5α-androstane has a greater impact on affinity than the presence of only a 17β-hydroxyl group. However, both of these functional groups have similar impacts upon the affinity of 5α-androstane for AR2.

The effect of specific functional groups at the 3- and 17-position upon the affinity of androgens for AR1 and AR2 is similar to what has been shown for rat AR where Ojasoo et al. [31] found that the 17β-hydroxy,3-keto configuration is necessary for high affinity binding. Within the ligand binding domain of mammalian ARs there is a key interaction between a specific amino acid which acts as a strong hydrogen bond donor and the 3-keto group of androgens which acts as a strong hydrogen bond-acceptor [32]. Whether there is an analogous site within the ligand binding domain of fish ARs has yet to be determined. Ekena et al. [32] suggested that one of the distinguishing binding characteristics of the AR and ER is that the former recognizes the 3-keto group of most androgens, as opposed to the 3-hydroxyl group of estradiol, whereas, the 17β-hydroxyl group acts as a hydrogen bond donor to the ligand binding domains of both ARs [33] and ERs. [34].

Various 11-oxygenated androgens showed marked differences in their ability to bind to Atlantic croaker AR1 and AR2. With the exceptions of 11-KA and 11-KDHA the 11-oxygenated androgens showed little affinity for AR1 (see also [1]). The 13% RBA of 11-KA for AR1 suggests that this androgen could have physiological activity in vivo, however, the physiological importance of this androgen, as well as the other 11-oxygenated androgens, is not well understood. In contrast, 11-KT, and in particular 11-KDHT, bind to AR2 with relatively high affinity. 11-KT has low affinity for AR1-like receptors in both goldfish (*Carassius auratus*) [20] and in rainbow trout (*Oncorhynchus mykiss*) [21]. However, 11-KT has a greater affinity for the AR2-like receptor in coho salmon (*Oncorhynchus kisutch*) [22], but the 17% RBA of 11-KT for kelp bass AR2 [18] is similar to that of Atlantic croaker AR2 [1]. This suggests that in those fish where 11-KT is the dominant male androgen, the actions of 11-KT will be mediated via AR2 rather than AR1.

While it has been the generally accepted paradigm that 11-KT is the male-specific androgen responsible for inducing certain male-specific traits in fishes such as gonadal differentiation [13], spermatogenesis [14] and the induction of secondary sexual characteristics [15–17], there is considerable diversity in both the concentrations and the specific 11-oxygenated androgens produced within teleosts [9,10]. Males of some species have high circulating levels of 11-KT (e.g. [35]), whereas others also have high levels of 11β-HT (e.g. [36]). In addition, in species such as the urohaze-goby the testes do not produce 11-oxygenated steroids; instead they produce 5α-reduced androgens [27]. Binding studies in Atlantic croaker [1] and in kelp bass [18] demonstrate that other physiologically important androgens such as T and DHT have higher affinities for AR2 than 11-KT, suggesting that androgens other than 11-KT may be important in mediating the androgenic response within specific target tissues. This is particularly evident in females where 11-KT levels are generally low or non-existent [9], but AR2 is present in ovarian and brain tissue [1].

The biphasic binding curves of 11-KA and 11-KDHA suggest that there are negative allosteric effects on the binding affinity of these two steroids for AR2. Similar biphasic binding curves were shown for certain bivalent ligands binding to the lamb ER (lER) [37] and for the antiandrogen RU 59063 binding to the human AR (hAR). For both AR2 and the lER [37] the biphasic curves were found both with and without

molybdate present, whereas the binding of RU 59063 to the hAR was monophasic in the absence of molybdate [37,38]. Molybdate stabilizes the large 8–9S protein complex formed between heat shock and other chaperone proteins and steroid receptors [39], including AR2 [1]. Bergmann et al. [37] proposed a dimeric receptor model to explain their results, whereas Teutsch et al. [38] suggested a two-site model where one site induces a low affinity conformation within the second site, but they could not determine whether the sites were on the same or separate receptors. It is generally believed that receptors interact with heat shock proteins as monomers and form activated dimers only after ligand binding [39,40]. Moreover, as Teutsch et al. [38] indicated there is a lack of information surrounding the sequence of events of ligand binding and receptor activation and it is not known whether receptor–receptor interactions occur when heat shock proteins are bound to the receptors. Possible multiple binding sites on nuclear steroid receptors have been found for the glucocorticoid receptor [41] and PR [42]. Conversely, there are also reports of cooperative binding of estradiol to the ER, a phenomenon that suggests that the ER is a dimer prior to ligand binding [43].

The differences between the binding affinities of the synthetic steroids for AR1 and AR2 highlight the differences between these two receptors as well as similarities between AR2 and mammalian ARs. The addition of methyl groups and/or the removal of the 19-methyl group from T has deleterious effects upon the RBA of these steroids for AR1. However, the presence of a 7α-methyl group did not appear to influence the binding affinity for AR1, since 19-norT and mibolerone (17β-hydroxy-7α,17α-dimethyl-4-estren-3-one) [1] had similar binding affinities to 17α-M-norT. This suggests that the 7-position of T does not have a role in determining the specificity of the AR1 binding site. In general, the synthetic steroids have low affinity for AR1 and R1881 whereas MDHT [1] and the five antiandrogens that were tested demonstrated no affinity. This is in direct contrast to the binding of these steroids to AR2. Mibolerone [1], R1881, MT and MDHT all have high affinity for AR2, which is nearly identical to the binding studies of the rat AR, where 19-norT, MT, MDHT, 17α-M-norT, 7α-M-norT and R1881 each bound with an RBA of at least 100% of DHT [31]. In addition, the steroidal antiandrogen cyproterone acetate binds to AR2 with similar affinity to that of mammalian AR [44]. The fact that the non-steroidal antiandrogens RU 56187 and RU 3786 had RBAs within two and three orders of magnitude of DHT suggests that the binding site of AR2 possesses similar characteristics to the mammalian ARs for which these antiandrogens were developed.

In conclusion, few androgens bind to AR1 with high affinity and in general modifications to the basic 3-ketone,4-ene,17β-hydroxy structure of T reduce its binding affinity for AR1, suggesting that there is a strict conformational requirement for high affinity binding to AR1. However, certain androgens with ketone groups at the 3- and 17-position also bind with high affinity to AR1, suggesting that there may be several high affinity conformations that AR1 can occupy depending upon whether an androgen possesses a ketone or a hydroxyl group at the 17-position. The binding of androgens to AR2 shows a more predictable pattern with 5α-reduced steroids binding better than 4-ene steroids and substitutions at any position to the basic 3-keto,17-hydroxy motif of DHT lowering the binding affinity of a steroid. However, structural changes do not always prevent high affinity binding and, as a result, AR2 possesses a broader affinity for androgens than AR1 and a greater affinity for structurally diverse androgens.

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