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Cytosolic glucocorticoid receptor in the testis of *Bufo arenarum*: Seasonal changes in its binding parameters

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Abstract

Glucocorticoids (GC) are the hormonal mediators of stress. In mammals, high levels of GC have negative effects on reproductive physiology. For instance, GC can inhibit testicular testosterone synthesis by acting via glucocorticoid receptors (GR), the extent of the inhibition being dependent on GC levels. However, the effect of GC on testicular function and even the presence of GR in amphibians are still unclear. The purpose of this work was to characterise testicular cytosolic GR in *Bufo arenarum*, determining the seasonal changes in its binding parameters as well as the intratesticular localisation. The binding assays were performed in testis cytosol with [^{3}H]dexamethasone (DEX) and [^{3}H]corticosterone (CORT). Binding kinetics of DEX and CORT fitted to a one-site model. Results were expressed as means ± standard error. Apparent number of binding sites (Bapp) was similar for both steroids (Bapp DEX = 352.53 ± 72.08 fmol/mg protein; Bapp CORT = 454.24 ± 134.97 fmol/mg protein) suggesting that both hormones bind to the same site. Competition studies with different steroids showed that the order of displacement of [^{3}H]DEX and [^{3}H]CORT specific binding is: DEX ~ RU486 ~ deoxycorticosterone (DOC) > CORT > aldosterone > RU28362 > progesterone >>> 11-dehydroCORT. The affinity of GR for DEX (K_d = 11.2 ± 1.5 nM) remained constant throughout the year while circulating CORT clearly increased during the reproductive season. Therefore, testis sensitivity to GC action would depend mainly on inactivating mechanisms (11β-hydroxysteroid dehydrogenase type 2) and CORT plasma levels. Since total and free CORT are higher in the reproductive than in the non-reproductive period, the magnitude of GC actions could be higher during the breeding season. The intratesticular localisation of the GR was determined after separation of cells by a Percoll density gradient followed by binding assays in each fraction. DEX binds to two different fractions corresponding to Leydig and Sertoli cells. In conclusion, in the testis of *B. arenarum* GC could regulate the function of both cellular types particularly during breeding when CORT reaches the highest plasma concentration.

Keywords: Glucocorticoid receptor; Glucocorticoids; Intratesticular localisation; Testis; Toad

1. Introduction

Glucocorticoids (GC) exert a wide variety of actions in virtually all organs, regulating metabolism, growth, development, and neuronal function. In vertebrates, GC interact with at least two intracellular receptors named type I or mineralocorticoid receptor (MR) and type II or glucocorticoid receptor (GR), the majority of GC effects occurring via the GR (Funder, 1997). Additionally, rapid non-genomic actions mediated by plasma membrane-associated receptor have been proposed for several species (Borski, 2000). In amphibians, intracellular as well as membrane-associated receptors for GC have been described in liver, kidney, and skin cytosol (Lange and Hanke, 1988; Lange et al., 1988; Orchinik et al., 2000) and neuronal membranes (Evans et al., 2000; Orchinik et al., 2000, 1991).

Among the great variety of actions, GC potently disrupt mammalian reproductive physiology through a number of mechanisms. Several studies suggest that basal concentrations of GC are insufficient to disrupt reproductive physiology

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(Sapolsky et al., 2000). Traditionally, GC-mediated stress is thought to inhibit reproduction at several levels not only in mammals (Sapolsky et al., 2000) but also in amphibians (Moore et al., 2005). In male *Taricha granulosa*, amplectic-clasping behaviour is rapidly suppressed by corticosterone (CORT) or after exposing males to stressful conditions that stimulate CORT secretion (Moore and Miller, 1984; Moore and Zoeller, 1985). However, it is evident that during breeding many amphibians display a significant elevation of GC levels with no suppression of reproductive behaviour or physiology. Therefore, it is possible that elevated plasma levels of GC facilitate several aspects of reproduction. Moreover, studies in amphibians report a positive correlation between CORT and reproduction (Moore and Jessop, 2003; Romero, 2002) although there is a lack of information regarding the effect of GC on testicular function.

In rat Leydig cells, GC can inhibit testosterone production (Monder et al., 1994a) through its interaction with GR (Monder et al., 1994b). However, rat Leydig cells express a predominantly oxidative 11β-hydroxysteroid dehydrogenase (11β-HSD) activity, which protects the testis from GC effects (Gao et al., 1996, 1997). The question now arises as to the fate of the testicular function during breeding in amphibians with high GC concentrations (Romero, 2002). In the testes of *B. arenarum*, the presence of an 11β-HSD with oxidative activity has been demonstrated (Denari and Ceballos, 2005). This enzyme could modulate the amount of active CORT in testis cells, protecting this tissue from the potentially negative effect of GC on steroidogenesis. However, an increase of CORT due to stressful situations could overcome 11β-HSD protection, decreasing gonadal steroid synthesis as previously described in mammals (Monder et al., 1994a).

Although in amphibians the activity of 11β-HSD protects Leydig cells from the effect of high levels of GC, the sensitivity once GC reaches these cells is also important. For instance, annual changes in GC concentrations and 11β-HSD activity could be accompanied by compensatory changes in GR at the Leydig cell level. The amount of GR is known to change in laboratory studies, altering GC physiological effects (Munck and Náray-Fejes-Tóth, 1992). For example, if the increased baseline and stress GC concentrations during breeding were associated to a decrease in GR number or affinity, then the net physiological response might be identical during each season. However, the presence of a testicular GR as well as a seasonal study of its binding parameters is required to ascertain that hypothesis. This paper characterises a cytosolic GR in the testis of *B. arenarum*, describing seasonal changes in its binding parameters as well as its intratesticular localisation.

2. Methods

2.1. Materials

Dexamethasone (DEX), corticosterone (CORT), 11-dehydrocorticosterone (11-dehydroCORT), aldosterone, RU486, RU28362, progesterone, deoxycorticosterone (DOC), testosterone, and MS222 were from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]CORT (70.5 Ci/mmol), [^{3}H]DEX (35 Ci/mmol), and [^{3}H]testosterone (75.5 Ci/mmol) were from NEN (Boston, MA, USA). Percoll density marker beads were from Sigma Chemical (St. Louis, MO). Human chorionic gonadotropin (hCG) was from Elea Laboratory (Buenos Aires, Argentina) and human recombinant FSH were from Serono Laboratory (Spain). Testosterone antibody was from the Colorado State University and the kit for cAMP determination was from DPC Laboratory (Los Angeles, CA). All other chemicals were of reagent grade.

2.2. Binding assays

Testicular tissue was obtained from male toads *B. arenarum* collected in the neighbouring area of Buenos Aires throughout the year. Toads were kept at 25 °C for two days prior to use. Animals were deeply anaesthetised by placing in 0.5% aqueous solution of MS222. Testes were quickly removed, carefully separated from Bidder's organ and cut into small pieces. Fragments were rinsed repeatedly in ice-cold GR buffer (10 mM Hepes buffer with 5 mM EDTA, 10% (v/v) glycerol, 20 mM Na_2MoO_4, and 0.1 mM PMSF, pH 7.4) and homogenised (650 mg of tissue/ml) in the same buffer. Cytosolic fraction was prepared from the homogenate by differential centrifugation (Pozzi et al., 1997). After sedimentation of the nuclear fraction at 800*g* for 10 min, cytosolic fraction was separated from mitochondria and microsomes by centrifugation at 105,000*g* for 60 min. All steps were carried out at 4 °C. Protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin as standard. Binding was assayed in triplicate employing 400–600 μg cytosolic proteins and 3.5 nM [^{3}H]DEX or [^{3}H]CORT in GR buffer. Cytosolic fractions were prepared from testes of 4–8 animals each experiment. Binding parameters were obtained by the displacement of [^{3}H]DEX or [^{3}H]CORT specific binding with different concentrations of unlabelled DEX or CORT (2.5–1000 nM) or the corresponding competitor: RU486 (mammalian GR antagonist), RU28362 (mammalian GR agonist), DOC, aldosterone, progesterone, and 11-dehydroCORT. All the incubations were carried out in a final volume of 0.5 ml at 4 °C. After equilibrium was reached unbound [^{3}H]DEX or [^{3}H]CORT was removed by the incubation with an equal volume of charcoal–dextran (2%:0.2%) in PBS, pH 7.4, during 20 min and subsequent centrifugation. Specific binding was calculated by subtracting non-specific binding obtained in parallel samples after the addition of a 1000-fold excess of unlabelled DEX or CORT. Binding parameters dissociation constants (K_d) and the number of binding sites (B) were obtained employing the Ligand Programme (Ligand Software David Rodbard, NIH). In amphibians, interrenals are deeply associated with renal tissue making endogenous GC removal by adrenalectomy not possible. Consequently, B values were informed as apparent number of binding sites (Bapp). Bapp refers to the total amount of free receptors depending on circulating GC. All the determinations were carried out at the same time of the day to avoid the influence of daily variations.

2.3. Intratesticular localisation

Intratesticular localisation of GR was determined after cells separation by a Percoll density gradient according to Pozzi et al. (2001) followed by binding assays in each fraction. In each experiment, testicular tissue from 4 to 6 animals obtained as described above was cut into small pieces and incubated in L_{15} medium with 10 mM Hepes (1 g/30 ml) containing 0.5 mg collagenase/ml and 0.02 mg DNAse/ml, pH 7.4, in a shaking water bath (90 cycles/min, 30 min) at 35 °C. After incubation, dispersed tissue was filtered through a nylon mesh and the remaining tissue was incubated for 20 min with fresh medium. Cell suspension was centrifuged (200*g*, 10 min) and washed twice with collagenase-free medium. Cells were re-suspended in L_{15} medium (approximately 50×10^6 cells/ml) and layered on a discontinuous Percoll gradient prepared with increasing concentrations of Percoll (20–70%) in L_{15} medium (Benahmed et al., 1985; Lefèvre et al., 1983; Pozzi et al., 2001). Red blood cells and spermatozoa were previously separated using Percoll 70%. Gradients were centrifuged at 800*g* for 25 min at room temperature. Fractions (1 ml) were collected from the bottom, washed and suspended in GR buffer. Linearity of each gradient was verified using den-

sity marker beads. Binding assays were carried out in each fraction after cellular lysis using [^{3}H]DEX as described above.

Leydig cells were characterised by their capacity to produce testosterone after hCG-treatment. For this purpose, 5×10^5 cells/ml of each fraction were incubated with or without 10 ng/ml hCG for 90 min at 35 °C in L_{15} medium. After incubations, media were frozen at −20 °C until testosterone determination. Sertoli cells were located by their capacity to produce cAMP after FSH-treatment: 5×10^5 cells/ml of each fraction were incubated with or without 10 ng/ml of FSH and 0.5 mM of isobutylmethylxanthine (phosphodiesterase inhibitor) for 30 min at 35 °C in L_{15} medium according to Gorczynska et al. (1996). After incubation, media and cells were frozen at −20 ° until cAMP determination. cAMP production was determined using a binding protein assay. Cells were frozen and thawed twice, centrifuged (1500*g*, 10 min) and supernatants assayed for cAMP. This method is very specific for cAMP with a low cross-reactivity with cGMP (0.1%). The sensitivity of the assay was 0.11 pmol. Samples were assayed in triplicate. Testosterone content in incubation media was determined by radioimmunoassay according Pozzi and Ceballos (2000). The cross-reactivity of testosterone antibody with dihydrotestosterone was 35%. The sensitivity of the assay was 6 pg/ml. Steroids were assayed in triplicate. Intra and inter-assay coefficients of variation were under 8 and 12%, respectively.

2.4. Corticosterone radioimmunoassay

Blood samples were obtained from the heart with heparinised syringes and collected in heparinised tubes. Samples were centrifuged at approximately 600*g* for 10 min, and plasma was removed and stored at −20 °C for future determinations. Plasma CORT levels were measured using radioimmunoassay as previously described (Denari and Ceballos, 2005). Briefly, plasma was extracted twice with ethyl ether, evaporated and re-dissolved in 100 mM borate buffer, 0.1% gelatine, pH 8.0. Losses during the entire procedure were calculated in parallel samples by adding [^{3}H]CORT. The antiserum against CORT was used in a final dilution of 1:22,500. The cross-reactivity with aldosterone was less than 0.1% (Gomez-Sanchez et al., 1975). The sensitivity of the assay was 50 pg/ml. Intra and inter-assay coefficients of variation were under 8 and 12%, respectively. Steroids were assayed in triplicate.

2.5. Radioactivity

The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac Co, Turku, Finland).

2.6. Statistical analysis

Results were expressed as means ± standard error. Binding parameters K_d and Bapp were obtained employing the Ligand Programme (Ligand Software David Rodbard, NIH). Non-transformed data were tested for one-site or two-site models. One-way ANOVAs followed by Tukey's multiple comparisons tests were used to detect significant differences among seasons in GR binding parameters, K_d and Bapp as well as in CORT concentrations. Differences were regarded as significant at $P < 0.05$. Before statistical analysis data were tested for normality and homoscedasticity using Lilliefors and Bartlett's tests, respectively.

3. Results

3.1. Preliminary results

The rate of association of [^{3}H]DEX and [^{3}H]CORT with cytosolic testicular proteins was examined by measuring specific binding at 4 °C after incubation times of 6–24 h. Apparent equilibrium was reached within 14–18 h and was stable until at least 24 h (data not shown). Subsequent competition assays were performed at 4 °C during 18 h.

3.2. Binding parameters of GR

Equilibrium competition analyses were conducted by incubating cytosolic proteins from toad collecting during the post-reproductive season (January to April) with 3.5 nM of either [^{3}H]DEX or [^{3}H]CORT and increasing amounts of the corresponding unlabelled steroids. Representative competition curves and Scatchard plot for both steroids are shown in Fig. 1. Binding kinetics of DEX as well as CORT fitted to a one-site model with K_d value higher for CORT (31.33 ± 2.68 nM) than for DEX (13.68 ± 3.01 nM). Bapp was similar for both steroids (Bapp DEX = 352.53 ± 72.08 fmol/mg protein; Bapp CORT = 454.24 ± 134.97 fmol/mg protein) suggesting that both hormones bind to the same site.

After determining the presence of GR in testicular cytosol, binding parameters were measured throughout the year using DEX as radioactive ligand. Results were initially assembled into the three different periods previously described for *B. arenarum* (Denari and Ceballos, 2005): pre-reproductive (Pre-R) from May to August, reproductive (R) from September to December, and post-reproductive (Post-R) from January to April. Pre-R and Post-R periods correspond to that previously defined as the non-reproductive period in this species (Canosa and Ceballos, 2002). As can be seen in Table 1 the affinity of GR remains constant throughout the year while circulating CORT clearly increases during the R season suggesting that toad testis exhibits a higher sensitivity to GC during the breeding. Table 1 also shows that Bapp is significantly different only between Pre-R and Post-R seasons. Fig. 2 shows representative saturation and Scatchard plots for all seasons.

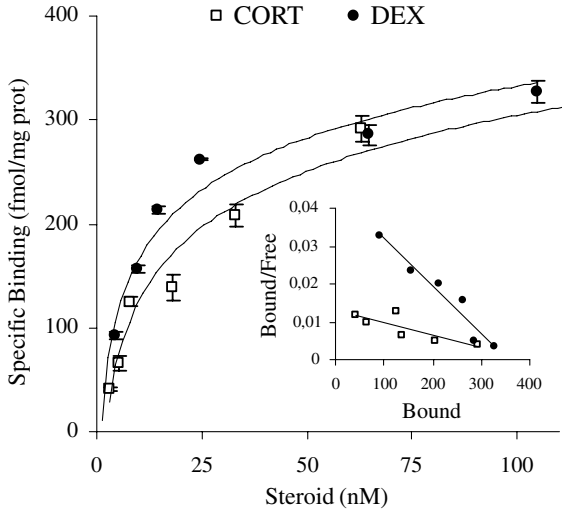

Fig. 1. Representative equilibrium saturation curve of binding of DEX and CORT in cytosol prepared from testes of four toads collected during the Post-R season. Error bars correspond to standard deviations. Experiments were repeated 5 and 9 times for B and DEX curves, respectively. The inset shows the Scatchard plot.

Table 1
Seasonal binding parameters of GR and plasma CORT concentrations

Period	K_d (nM)	Bapp (fmol/mg protein)	CORT (nM)
Pre-R	7.83 ± 0.91	197.63 ± 23.91	61.60 ± 10.34
R	12.49 ± 3.78	244.30 ± 24.15	144.59 ± 17.67*
Post-R	13.68 ± 3.01	339.18 ± 58.05*	95.70 ± 10.16

Binding parameters were obtained by the displacement of [^{3}H]DEX (3.5 nM) specific binding with different concentrations of unlabelled DEX (2.5–1000 nM). Experiments were performed with 400–600 μg cytosolic proteins in GR buffer. K_d and Bapp were calculated using the Ligand programme. Values represent means of 9 (Pre-R), 6 (R), and 9 (Post-R) independent experiments ± SE. *Significant differences with Pre-R period with $P < 0.05$. Plasma total CORT concentrations were obtained by RIA after ethyl ether extraction. Values are expressed as means of 25 (Pre-R), 26 (R) and 30 (Post-R) toads ± SE. *Significant differences with Pre-R and R periods with $P < 0.05$.

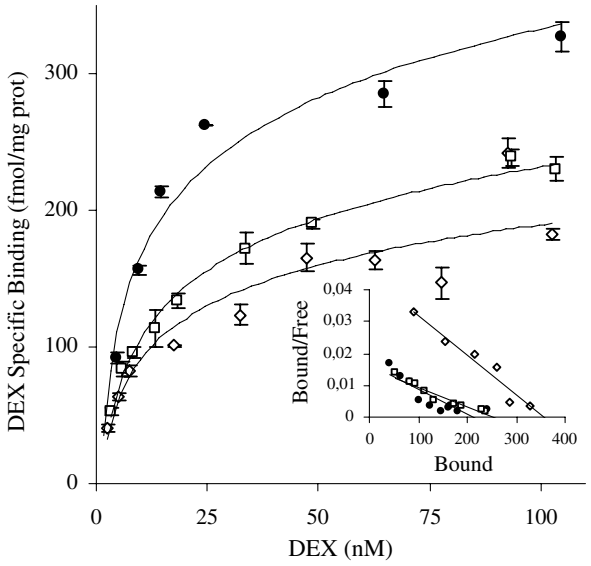

Fig. 2. Binding of DEX in testicular cytosol from toads collected during Pre-R (◇), R (□), and Post-R (●) seasons. Representative saturation curves and the corresponding Scatchard (inset) selected from 9 curves of Pre-R, 6 of R, and 9 of Post-R. Cytosol fractions were obtained from four toads for each curve. Error bars correspond to standard deviations.

3.3. Pharmacological characterisation of GR

To characterise the pharmacological properties of testicular GR, the specific binding of [^{3}H]DEX and [^{3}H]CORT to cytosol was displaced with different competitors. Competition studies with different compounds show that the order of displacement of [^{3}H]DEX (Fig. 3) specific binding is as follows: DEX ~ RU486 ~ DOC > CORT > aldosterone > RU28362 > progesterone >>> 11-dehydroCORT. Fig. 4 shows that a similar displacement rank is obtained when [^{3}H]CORT is used as a labelled ligand, suggesting that both steroids bind to the same receptor and display similar properties. Table 2 shows the K_i values for the steroids employed in the competition experiments with [^{3}H]DEX and [^{3}H]CORT.

3.4. Intratesticular localisation of GR

To determine the localisation of GR within the testis, cells were separated by a discontinuous Percoll gradient followed by binding assays in each fraction. After submitting crude-cell preparation to a discontinuous gradient, different cells were separated according to their densities. Coloured density marker beads were used to verify the linearity of the gradient, allowing the estimation of the density of each cellular fraction. Inset in Fig. 5 shows a representative example of the distribution of density markers. When cell viability was analysed by trypan-blue exclusion damaged cells showed to be located on the top of each gradient. DEX binds to two separated groups of fractions exhibiting densities of 1.050 g/ml (fractions 13–14) and 1.038 g/ml (fractions 18–19), corresponding to Leydig and Sertoli cells, respectively (Pozzi et al., 2001). Leydig and Sertoli cells were characterised for their capacity to produce testosterone and cAMP after stimulation with hCG and FSH, respectively. Fig. 5 shows a representative experiment.

4. Discussion

There are several determinants in setting the level of biological action of GC in testicular cells. Undoubtedly, plasma GC level is an important issue to take into account, particularly in cold-blooded vertebrates having pronounced changes in this parameter. In amphibians, an increase in GC concentration during the breeding season is clearly stated in several species (for review, see Romero, 2002). However, there are other components determining the sensitivity of one particular cell to GC. For example, an increased expression of 11β-HSD in target tissues could decrease the local GC concentration to an insufficient level to activate GR. Alternatively or concomitantly, an increased number and affinity of GR could increase the sensitivity to GC. On the other hand, also the abundance of plasma binding proteins could be involved in the regulation of the cellular response to GC. Corticosteroid binding globulin (CBG) binds GC with high affinity and therefore could regulate the availability of CORT. This binding of CORT to CBG may serve as a tissue buffer against potentially deleterious effects of elevated CORT, regulate the availability of free hormone to target tissues, or alter CORT clearance rates (Breuner and Orchinik, 2002; Yding Andersen, 2002). In mammals, several studies have demonstrated that CBG has high affinity for CORT and low affinity for androgens (Breuner and Orchinik, 2002). In few amphibian species (Martin and Ozon, 1975; Orchinik et al., 2000), CBG has similar or higher affinity for androgens than for B. Those results suggest that if T increases and CBG remains constant, B could be displaced from CBG resulting in an increase in free GC. In *B. arenarum*, the proportion of free B is highest during the Pre-R season (82%) and lowest during breeding (65%) when testosterone reaches its highest and lowest concentration, respectively (Denari and Ceballos, 2005). In consequence, it is possible to suggest that the

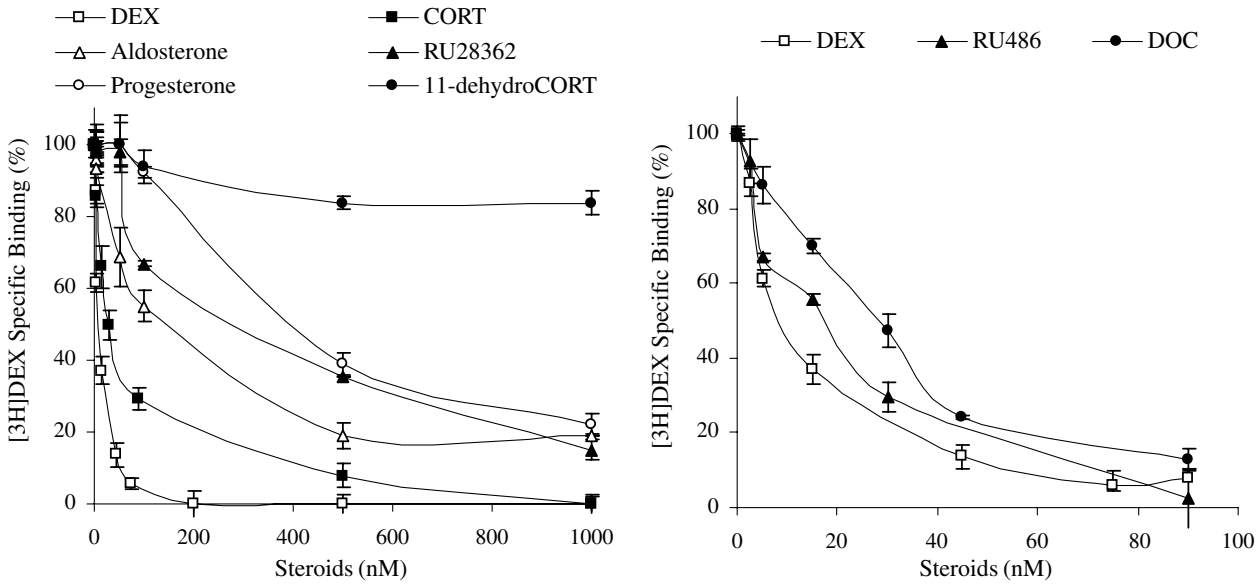

Fig. 3. Displacement curves of specific binding of [^{3}H]DEX by various steroids. Testes of six toads were employed in each experiment. Error bars correspond to standard deviations. Experiments were repeated 3 times. K_i values are given in Table 2.

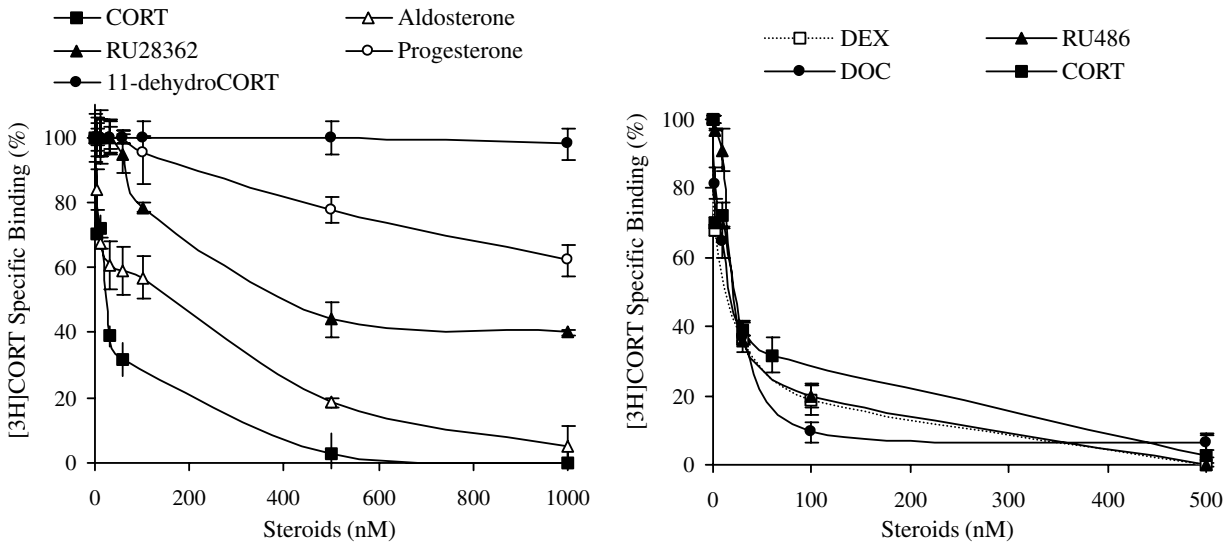

Fig. 4. Displacement curves of specific binding of [^{3}H]CORT by various steroids. Testes of six toads were employed in each experiment. Error bars correspond to standard deviations. Experiments were repeated 3 times. K_i values are given in Table 2.

increase in free B observed in *B. arenarum* during the Pre-R season could be due to a competition between androgens and B. Preliminary results from our laboratory indicate that testosterone displaces the binding of radioactive B with similar potency than B. But, a complete characterisation of CBG binding properties is necessary to assess that hypothesis.

Undoubtedly, amphibians are an interesting model to study the interaction of all those parameters mentioned before, particularly because of the pronounced metabolic changes they are exposed to throughout the year. *B. arenarum* is a seasonal breeder with a breeding season restricted to the period between September and December, i.e., springtime. Moreover, this species is characterised by having an androgen-dissociated breeding pattern (Canosa and Ceballos, 2002). Recently, it was demonstrated that the GC inactivating enzyme, 11β-HSD, is expressed in Leydig cells of the toad (Denari and Ceballos, 2005). During the post-reproductive season total 11β-HSD activity increases almost 10 times in comparison with the breeding season, suggesting that testicular cells could be less sensitive to GC during that season. On the other hand, during breeding there is an increase in GC concentration and a decrease in the amount of active 11β-HSD allowing to hypothesise that only a decrease in GR could avoid GC action in Leydig cells. However, a seasonal study of binding parameters of the testicular GR was required to ascertain that hypothesis.

Table 2
Steroid displacement of [^{3}H]DEX and [^{3}H]CORT from binding sites

Competitor	K_i (nM) [^{3}H]DEX	K_i (nM) [^{3}H]CORT
DEX	11.2 ± 1.5	13.5 ± 9.3
RU486	12.8 ± 1.9	14.1 ± 3.7
DOC	12.5 ± 3.9	19.7 ± 15.0
CORT	36.5 ± 3.0	29.3 ± 5.6
Aldosterone	117.8 ± 13.7	125.4 ± 45.9
RU28362	194.3 ± 31.0	298.0 ± 165.8
Progesterone	405.7 ± 57.1	1383.4 ± 509.5
11-DehydroCORT	6125.4 ± 458.0	>10,000

Binding was assayed employing 400–600 μg cytosolic proteins and 3.5 nM [^{3}H]DEX or [^{3}H]CORT in GR buffer. K_i values were obtained by the displacement of [^{3}H]DEX or [^{3}H]CORT specific binding with different concentrations of unlabelled DEX, CORT, RU486, RU28362, DOC, aldosterone, progesterone, and 11-dehydroCORT (2.5–1000 nM). K_i values are expressed as means of three independent experiments ± SE.

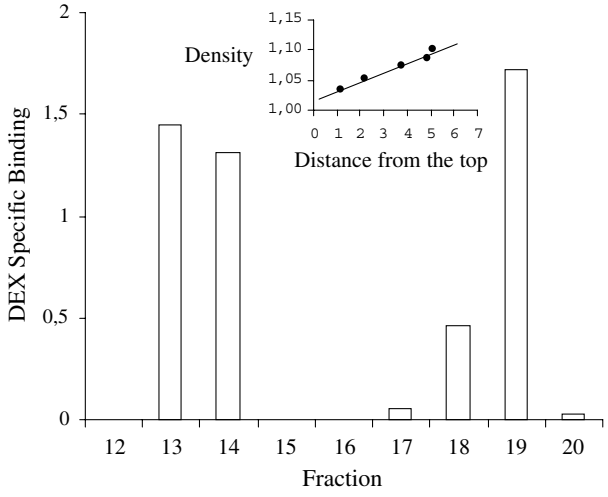

Fig. 5. Profile of DEX specific binding (pg/10^5 cells) in a Percoll gradient. Experiments were repeated 6 times and figure shows a representative experiment. Fractions 13–14 and 18–19 correspond to densities of 1.050 g/ml (Leydig cells) and 1.038 g/ml (Sertoli cells), respectively. For other details see Section 2. Inset: distribution of density marker beads in a typical Percoll gradient (20–70%). Plot shows the distance from the top (cm) of each band vs. the density of each band.

To start with, it was necessary to establish the presence of a GR in toad testicular cells. As in rat testes (Monder et al., 1994b), *B. arenarum* expresses a cytosolic receptor with higher affinity for DEX than for CORT. Dissociation constants higher for CORT than for DEX were also described for liver and brain cytosolic GR of *Ambystoma tigrinum* (Orchinik et al., 2000), liver of *Xenopus laevis* (Lange and Hanke, 1988), rat thymus (Vicent et al., 1999), and rat seminiferous tubules (Levy et al., 1989). However, in the toad the K_d for DEX is higher than values described for rat Leydig cells (Evain et al., 1976) and liver from other amphibians (Incerpi et al., 1983; Lange and Hanke, 1988; Orchinik et al., 2000; Woody and Jaffe, 1982). Like the mammalian GR, the toad receptor has high affinity for RU486 and DEX but almost 20 times less affinity for RU28362, a pure mammalian GR agonist (Quirk et al., 1986), than for DEX.

In addition, in agreement with the protective role of the 11β-HSD type 2 (Monder et al., 1994b), also present in toad testes (Denari and Ceballos, 2005), 11-dehydroCORT binds to GR with much less affinity than CORT. Unexpectedly, GR from toad testis binds with higher affinity DOC than CORT. In another amphibian, *A. tigrinum*, DOC has much less affinity for liver and brain cytosolic GR than CORT (Orchinik et al., 2000). However, in rat Leydig cells DOC increases testicular 11β-HSD through GR (Nwe et al., 1999). At present, in amphibians there is no evidence regarding an effect of DOC on testicular 11β-HSD.

As liver GR from male *X. laevis* (Lange and Hanke, 1988), there were non-significant differences among the dissociation constants for labelled DEX throughout the year. Since that binding parameter was constant all year long, the magnitude of the GC effect on toad testes could depend on variations in the amount of GR, 11β-HSD activity or in plasma B concentration.

Regarding Bapp, the apparent number of GR, the influence of endogenous GC on the receptor assay cannot be excluded. In mammals, the levels of endogenous GC are kept very low by adrenalectomy. In amphibians that procedure is technically impossible, determining that the method commonly employed can measure only unoccupied receptors. As a consequence, high levels of CORT cause an underestimation of the total receptor concentration. Assuming that the higher the CORT concentration the lower the free receptor concentration, it is possible to consider that during breeding, when CORT reaches the highest concentration, the underestimation would be higher than during the Pre-R season. During the Post-R season, the only season exhibiting an increase in Bapp, CORT concentration is lower than during breeding and this is probably the reason for the apparent increase in the amount of total receptors. Since the most important seasonal change is found in plasma B levels, the reproductive period would be the most sensitive one to GC action. Seasonal studies of GC testicular effects are required to test that idea. In *B. arenarum*, testosterone levels decrease during the breeding season (Canosa and Ceballos, 2002). It could be possible that the increase in B level during that period cause, at least in part, the decrease in testosterone plasma level.

Another important aspect to keep in mind refers to the intratesticular localisation of the GR and the association with 11β-HSD activity. In the toad, GR seems to be located in Leydig as well as Sertoli cells. Specific binging of labelled steroids was obtained in fractions with densities of 1.050 and 1.038 g/ml corresponding to Leydig and Sertoli cells, respectively (Pozzi et al., 2001). This dual localisation is comparable with intratesticular distribution in the rat (Evain et al., 1976; Levy et al., 1989). In rat Leydig cells, GC inhibit testosterone biosynthesis via the interaction with GR (Bambino and Hsueh, 1981; Monder et al., 1994a) while the physiological action on Sertoli cells remains still unknown. Since 11β-HSD type 2 decreases intracellular concentration of active GC, the co-expression of GR with 11β-HSD would be determinant of the sensitivity to GC of

the different cell types. Rat Leydig cells are capable of regulating the concentration of active GC through the expression of an 11β-HSD with oxidative activity (Gao et al., 1996, 1997), protecting these cells from the suppressive effect of GC on T production (Monder et al., 1994b). On the other hand, Sertoli cells would lack 11β-HSD, making them more sensitive to GC actions. Preliminary studies performed in *B. arenarum* indicate that 11β-HSD is located only in Leydig cells. However, seasonal studies are required to ascertain the role of GC on testicular function.

One possibility to take into account is that a type I-like receptor (MR) could be partially involved in GC action. However, preliminary studies suggest that is not possible to measure the binding of labelled aldosterone in the presence of RU486, aldosterone being able to bind to testicular cytosol only when type II-like GC receptor (GR) is not occupied by RU486. However, more studies are necessary to clearly determine the absence of a type I-like receptor in toad testes.

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