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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-