RESEARCH ARTICLE

Seasonal Changes and Regulation of the Glucocorticoid Receptor in the Testis of the Toad *Rhinella arenarum*

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ABSTRACT

Several studies indicate that wild free-living vertebrates seasonally regulate plasma glucocorticoids. However, not only glucocorticoids but also the amount of receptors is important in determining biological responses. In this context, seasonal regulation of glucocorticoid receptor (GR) is crucial to modulate the response to glucocorticoids. *Rhinella arenarum* is an anuran exhibiting seasonal variations in plasma glucocorticoids and also in the number of binding sites (B_{max}) of the testicular cytosolic GR. In this work, we evaluated if the annual pattern of GR protein in the testis varies seasonally and, by an in vitro approach, the role of glucocorticoids, androgens, and melatonin in the regulation of the GR B_{max} and protein level. For this purpose, testes were treated with two physiological concentrations of melatonin (40 and 200 pg/ml), with or without luzindole (melatonin-receptor antagonist); with testosterone, cyanoketone (inhibitor of steroidogenesis) or casodex (androgen-receptor antagonist); or with dexamethasone or RU486 (GR antagonist). After treatments, B_{max} and protein level were determined by the binding of [^{3}H]dexamethasone and Western blot, respectively. Results showed that GR protein decreases in the winter. The in vitro treatment with melatonin produced a biphasic effect on the B_{max} with the lowest concentration decreasing this parameter by a receptor-mediated mechanism. However, melatonin had no effect on the GR protein level. Conversely, a high concentration of dexamethasone up-regulated the GR protein and androgens neither changed the B_{max} nor the protein level. These findings suggest that seasonal changes in plasma melatonin and glucocorticoids modulate the effect of glucocorticoids in the testis of *R. arenarum*. *J. Exp. Zool. 319A:39–52, 2013.* © 2012 Wiley Periodicals, Inc.

J. Exp. Zool. 319A:39–52, 2013

How to cite this article: Regueira E, Sassone AG, Scaia MF, Volonteri MC, Ceballos NR. 2013. Seasonal changes and regulation of the glucocorticoid receptor in the testis of the toad *Rhinella arenarum*. J. Exp. Zool. 319A:39–52.

Glucocorticoids (GCs) are steroid hormones synthesized in vertebrate adrenal/interrenal gland that exert a wide variety of actions in virtually all organs. Particularly, stress mediated increase of plasma GCs potently suppress mammalian gonadal axis (Free and Tillson, '73; Matteri et al., '84; Sapolsky, '85)

Additional supporting information may be found in the online version of this article.

Grant sponsor: Universidad de Buenos Aires; Grant number: UBACYT X042; Grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas; Grant number: PIP 2433; Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica de la Argentina; Grant number: PICT 32219.

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Received 9 August 2012; Revised 14 September 2012; Accepted 1 October 2012

Published online 30 November 2012 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/jez.1772

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whereas in amphibians GCs disrupt reproduction by inhibiting the reproductive behavior (Moore and Jessop, 2003; Moore et al., 2005). However, in several amphibians and reptiles there are seasonal changes in baseline plasma GCs with higher concentrations during the breeding than during the non-reproductive season (Romero, 2002; Moore and Jessop, 2003). Hence, to further understand if GCs annual rhythms have any physiological consequence, several components determining the sensitivity of target tissues should be considered, among them, the number and affinity of the glucocorticoid receptor, the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) with inactivating/activating activity (Monder and White, '93; White et al., '97) and the amount of plasma GC-binding proteins (Breuner and Orchinik, 2001). However, data examining potential changes on these parameters are scarce for amphibians.

In vertebrates, GCs interact with at least two different intracellular receptors, the type I or mineralocorticoid receptor and the type II or glucocorticoid receptors (GR), the majority of GCs actions occurring via the GR (Funder, '97). Besides, rapid non-genomic actions mediated by plasma membrane-associated receptor were proposed for several species (Borski, 2000). The GR is a transcription factor located in the cytoplasm and the binding of GCs induces dissociation of the GR from heat shock proteins (hsp), dimerization, nuclear translocation and binding to DNA (Gross and Cidlowski, 2008). There is one gene that codes for GR in almost all vertebrates but multiple promoters, alternative splicing, alternative translation initiation, and posttranslational modifications result in the production of various isoforms with different distribution patterns and transcriptional regulatory profiles (Yudt and Cidlowski, 2002; Lu and Cidlowski, 2006; Oakley and Cidlowski, 2011).

Auto- and cross-regulation of nuclear receptors is an ancient and conserved regulatory mechanism for controlling cell responses to hormones (Bagamasbad and Denver, 2011). Autoinduction of the GR by GCs was demonstrated in human leukemic T cells (Eisen et al., '88; Denton et al., '93; Ramdas et al., '99) and in tadpole intestine (Krain and Denver, 2004), while down-regulation was described in mammals and in frogs and may be a more common form of autoregulation of the GR than autoinduction (Bagamasbad and Denver, 2011). Bagamasbad and Denver (2011) proposed that the cross-regulation of nuclear receptors could coordinate the responsiveness of a cell to a subsequent hormonal signal, or to allow for crosstalk between hormonal signaling pathways. Particularly androgens, down-regulate GR mRNA expression in prostate and epididymis of the rat (Rennie et al., '89; Silva et al., 2010), and in vivo treatments with testosterone or estrogen also decreases mRNA levels in the hippocampus (Burgess and Handa, '93; Kerr et al., '96). In amphibians, there are no studies regarding the effect of androgens on GR regulation.

Several reports propose a link between melatonin and GR activity. In mouse thymocytes melatonin down-regulates GR mRNA (Sainz et al., '99) and inhibits GR nuclear translocation by inhibiting the dissociation from the chaperone hsp 90 (Presman et al., 2006). In mouse hippocampal cells, melatonin prevents GCs inhibition of cell proliferation and toxicity by reducing GR nuclear translocation (Quiros et al., 2008). Also, physiological concentrations of melatonin impair GR interaction with the transcriptional intermediary factor 2 in newborn hamster kidney cells (Presman et al., 2012). In human leucocytes, melatonin increases the dissociation constant of the GR for GCs without affecting the number of receptors, suggesting that seasonal changes in melatonin concentration could produce seasonal variations in GR binding parameters (Blackhurst et al., 2001). In addition, in *Xenopus laevis* photoperiod alters the binding of radioactive corticosterone to the liver (Lange and Hanke, '88).

Rhinella arenarum is a South American toad with a breeding season restricted to springtime, that is, between September and December. This toad is characterized by having the highest concentration of plasma GCs and the lowest concentration of androgens during the breeding period (Canosa et al., '98; Canosa and Ceballos, 2002; Denari and Ceballos, 2005). In addition, concentration of GCs that correspond to the reproductive period inhibits the synthesis of testosterone in the testis by a GR mediated mechanism (Tesone et al., 2012). A cytosolic GR was detected in Leydig and Sertoli cells and the studies on the seasonal changes of GR parameters showed significant differences only in the apparent number of binding sites, being lowest during the pre-reproductive season (winter; Denari and Ceballos, 2006). However, it is not known if this variation in the number of binding sites is due to changes in the amount of the GR protein or to another component of the GR complex.

These results clearly indicate that to understand the consequence of seasonal changes in plasma GCs on testicular function, it is important to analyze changes in the number of the GR. The main goal of this article is to study the seasonal variation in the amount of GR protein as well as the role of androgens, melatonin and GCs in the regulation of the GR in the testis of the toad *R. arenarum*. For this purpose, the in vitro effect of melatonin, androgens and GCs on testicular GR was evaluated by analyzing changes in the GR number of binding sites and the amount of GR protein.

MATERIALS AND METHODS

Animals and Tissue Preparation

Testes were obtained from adult male toads *R. arenarum* (150–180 g) collected during the pre-reproductive (May to August), reproductive (September to December) and postreproductive (January to April) periods described by Denari and Ceballos (2005), in the neighboring area of Buenos Aires city (Argentina). Animals were maintained under natural conditions with free access to water and fed with crickets and zophobas. Toads were over-anesthetized by immersion in 1% aqueous solution of MS222

(Sigma-Aldrich, St. Louis, MO, USA). Testes were quickly removed, placed in Petri dishes with sterile DMEM medium containing 10 mM HEPES, 10% charcoal-stripped fetal bovine serum, antibiotic (penicillin, 100 IU/ml, streptomycin, 0.1 mg/ml) and antimycotic (amphotericin B, 0.25 mg/ml), and then carefully separated from the Bidder's organ. The experiments comply with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction and with the approval of the Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina. All efforts were made to minimize the number of animals used and their suffering.

Validation of Antibody Specificity and Translocation Experiment

The specificity of the antibody raised against mouse receptor for the determination of the GR protein of *R. arenarum* was validated by pre-incubation of the antibody with the corresponding blocking peptide (200 μg/ml of immunization peptide, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hr. To further assess if the GR protein bands detected with the antibody were cytoplasmic isoforms or a plasma membrane associated GR, cytoplasmic and plasma membrane-enriched fractions were separated by differential centrifugation, as explained below, and analyzed by Western blot. The capacity of the isoforms to translocate to the nucleus was studied by a translocation experiment incubating testicular slices with 500 nM dexamethasone (Dex) for 1 hr. After incubation with Dex, nuclear and postnuclear fractions from the testicular homogenates were separated by differential centrifugation and submitted to Western blot for GR detection. The correct separation of the nuclear and postnuclear fractions was corroborated by the analysis of Western blot of a nuclear marker, proliferating cell nuclear antigen (PCNA), in both fractions.

Seasonal Variation in GR Proteins

Testes from animals collected during the postreproductive, prereproductive, and reproductive seasons were obtained as previously mentioned. Testes from the same animal were pooled and homogenized with ice-cold GR buffer (10 mM HEPES, 5 mM EDTA, 10% (v/v) glycerol, 20 mM Na_2MoO_4 and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) employing a manual Teflon-glass Potter-Elvehjem homogenizer. Samples were centrifuged at 400*g* for 10 min to sediment unbroken cells and supernatants were named "cell homogenate." Each homogenate was submitted to Western blot analysis and immunoreactive bands were semi-quantified.

Long-Term In Vitro Incubations

An in vitro approach was chosen to evaluate the role of androgens, melatonin, and GCs on the regulation of the GR. For each replicate, testes from different animals were cut into homogeneous slices of about 2 mm and, since binding assay requires large quantities of proteins, slices from different testes were pooled. Then, the slices were randomly separated into different groups. With this design each testis was equally represented in each of the incubation conditions. One group was immediately processed to check the initial GR binding capacity of the testes while the other groups were incubated for 24 hr at 28°C in Petri dishes containing either 10 ml medium alone (control) or with one of the corresponding treatments. As the half-life of the GR protein is between 8 and 20 hr, a 24 hr incubation time was selected in order to detect any decrease in the GR protein synthesis (Hoeck et al., '89; Webster et al., '97). All the experiments described in the following sections were repeated several times in both the reproductive and the non-reproductive seasons. The number of replicates made for each experiment is indicated in the respective figure legend. All the hormones and antagonists were purchased from Sigma-Aldrich. CNK was from Sterling–Winthrop Laboratory. Steroids, CNK, casodex, melatonin, and luzindole were dissolved in ethanol and diluted with the incubation medium to a final concentration of 1%. Control incubations also included 1% ethanol. Melatonin and luzindole were prepared immediately before incubations.

Role of Androgens on GR B_{max} and Protein. For determining the role of androgens in the regulation of the GR, testes from four animals were pooled for each replicate. Slices were submitted to one of the following treatments: (1) 10^{-7} M testosterone or 10^{-7} M 5α-dihydrotestosterone (DHT), to evaluate the effect of the addition of exogenous androgens; (2) 10^{-5} M of the androgen-receptor antagonist, casodex or 10^{-5} M cyanoketone (CNK) to analyze the effect of the inhibition of endogenous androgens. The concentration of CNK employed guarantees the maximum inhibition of the key enzyme for steroid biosynthesis, the 3β-hydroxysteroid dehydrogenase/isomerase (Pozzi and Ceballos, 2000). The amount of casodex corresponds to the concentration that displaces 95% of testosterone binding to its receptor (unpublished data from our laboratory). The concentrations of testosterone and DHT used mimic those found during the pre-reproductive period of *R. arenarum* (Canosa et al., 2003; Denari and Ceballos, 2005). After incubations, culture media corresponding to control and CNK treatments were stored at −20°C until testosterone determination by radioimmunoassay. Slices from all the treatments were processed for binding assay and Western blot analysis.

Role of Melatonin on GR B_{max} and Protein. For these experiments, testes from five animals were pooled for each replicate. As mentioned above, testicular slices were separated into several groups: one group was used to check for the initial GR binding capacity of the testis and the others were incubated with either: medium alone (control), 40 or 200 pg/ml melatonin. Both concentrations of melatonin correspond to the lowest and the highest plasma levels found in *Rana perezi* during the summer (Delgado and Vivien-Roels, '89). The lowest concentration (40 pg/ml) was also found during the winter, when melatonin rhythm

seems to disappear (Delgado and Vivien-Roels, ’89; Delgado et al., ’93). The effect of luzindole, a non-specific antagonist of plasma membrane melatonin receptors (Dubocovich, ’88), was evaluated to discriminate whether the action of melatonin on the testicular GR was mediated by a receptor or a non-receptor mediated mechanism (Reiter et al., 2000). For this, testicular slices were incubated with: medium alone (control), 1 μM luzindole, 40 or 200 pg/ml melatonin in presence of 1 μM luzindole. After incubations, slices from all the treatments were processed for binding assay and Western blot analysis.

Role of Melatonin on the Affinity Constant of the GR. The effect of melatonin on the affinity of the GR was determined by competition experiments. Testes from six animals were pooled for each replicate and slices were incubated during 24 hr in fresh medium alone (control) or in the presence of 40 pg/ml melatonin. After incubation, slices were processed for competition experiment as described below. Only the effect of melatonin was evaluated in these experiments because it was the only hormone producing a significant effect on the GR number of binding sites after 24 hr incubation.

Role of GCs on GR Protein. For these experiments, testes from one animal were pooled for each replicate. In these experiments the effect of two concentrations of Dex, 15 and 150 nM, and of the GR antagonist, RU486, was evaluated. Dex concentrations correspond to plasma levels of corticosterone during the pre-reproductive and reproductive periods, respectively (Denari and Ceballos, 2005). Since the 11β-HSD enzyme has low affinity for Dex (Diederich et al., ’97), Dex was used instead of corticosterone to avoid the incidence of seasonal variations of the 11β-HSD enzyme in the testis (Denari and Ceballos, 2005). In addition, Dex concentrations were similar to plasma corticosterone because in the toad both steroids bind to the GR with similar affinity (Denari and Ceballos, 2006). The incubations with the antagonist RU486 were done to discriminate a GR mediated effect of GCs. The concentration of RU486, 1 uM, corresponds to the concentration that displaces the entire union of Dexa to the GR (Denari and Ceballos, 2006). Testicular slices were separated in six groups and incubated with either: medium alone (control), 1 μM RU486, 15 nM Dex, 150 nM Dex, 15 nM Dex plus 1 μM RU486, or 150 nM Dex plus 1 μM RU486. After incubations, slices from all the treatments were processed for Western blot analysis. The effect of GCs on GR B_{max} was not evaluated because the exogenous GCs interfere with the binding assay.

Short-Term In Vitro Incubations

In these experiments, 3-hr incubation was selected in order to detect any decrease in the GR binding capacity due to posttranslational modifications, including ubiquitination, sumoylation, acetylation, and phosphorylation, which play an important role in modulating the biological actions of the GR (Duma et al., 2006; Faus and Haendler, 2006). Only the effect of melatonin was evaluated in these experiments because this hormone produced a significant effect on the GR number of binding sites after 24 hr incubation without modifying the GR protein. Testes from three animals were pooled and processed as described above. Slices were incubated for 3 hr in medium alone (control) or in the presence of either 40 or 200 pg/ml melatonin. After incubation, testes were processed for the determination of the number of binding sites as described below.

Differential Centrifugation

After the corresponding incubation, testes were homogenized with ice-cold GR buffer (10 mM HEPES, 5 mM EDTA, 10% (v/v) glycerol, 20 mM Na_2MoO_4 and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) employing a manual Teflon-glass Potter-Elvehjem homogenizer. Samples were centrifuged at 400*g* for 10 min to sediment unbroken cells and supernatants were referred as cell homogenate. Postnuclear fraction was prepared from the homogenate by centrifugation at 800*g* for 10 min to sediment the nuclear fraction. Nuclear fraction was re-suspended in the GR buffer. To obtain a plasma membrane-enriched fraction, the postnuclear fraction was further centrifuged at 12,000*g* for 10 min and the pellet was re-suspended in the GR buffer. All steps were carried out at 4°C. Protein concentration was estimated by the method of Bradford using bovine serum albumin as standard (Bradford, ’76). Homogenates, postnuclear and nuclear fractions were used for the Western blot analysis, estimation of the number of binding sites and estimation of dissociation constants as indicated in the corresponding sections. The plasma membrane-enriched fraction was used to evaluate the presence of a plasma membrane GR in the testis as mentioned above.

Estimation of the Number of Binding Sites

Due to the fact that the receptor binding assay only measures non-activated free cytosolic GRs (Spencer et al., ’90), these assays were performed with the postnuclear fraction to minimize the non-specific interactions. The determination of the total number of binding sites was performed in triplicate by employing 1,000–1,600 μg of postnuclear proteins under saturating conditions (5 nM [^{3}H]Dex, 40 Ci/mmol, Perkin-Elmer, Boston, MA, MA, USA). All the incubations were carried out in a final volume of 0.5 ml at 4°C for 18–22 hr. After equilibrium was reached unbound [^{3}H]Dex was removed by incubation with an equal volume of charcoal-dextran (2–0.2%, w/v) in phosphate buffered saline, pH 7.4 and subsequent centrifugation. Specific binding was calculated by subtracting non-specific binding obtained in parallel samples after the addition of a 1,000-fold excess of unlabelled Dex.

In order to determine if melatonin decreased the binding of the GR by interfering the binding between the GR complex and the ligand, a set of binding assays were performed with postnuclear fraction of untreated fresh testis in the presence of melatonin (0.17, 0.52, 0.86 nM). The concentrations assayed are within the range of those employed for in vitro incubations.

Estimation of Dissociations Constant by Competition Experiment

Dissociation constants (K_d) were obtained by the competition of [^{3}H]Dex specific binding with different concentrations of unlabelled Dex (2.5–200 nM). Each concentration of unlabelled Dex was assayed in triplicate with 1,000–1,600 μg of postnuclear proteins from testes incubated with medium alone (control) or with 40 pg/ml melatonin. All the incubations were carried out in a final volume of 0.5 ml at 4°C for 18–22 hr. After equilibrium was reached, unbound [^{3}H]Dex was removed as described above.

Western Blot Analysis

To detect changes in the total amount of GR protein, semi-quantification was done in the homogenate. For this purpose, 100 μg of proteins were mixed with 40 mM Tris–HCl, supplemented with 2% sodium lauryl sulfate (w/v), 4% glycerol (v/v), 0.001% bromophenol blue (w/v), 2 mM β-mercaptoethanol, pH 6.8. Proteins were boiled for 5 min and centrifuged for 2 min at 12,000 g. Samples were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and electrophoresed for 140 min at 130 V. After electrophoresis, proteins were transferred to polyvinyl difluoride membranes (Hybond LFP, GE Healthcare Biosciences, Pittsburgh, PA, USA) at 300 mA for 90 min. After transference, membranes were incubated for 10 min in phosphate buffered solution containing 0.1% Tween-20 (PBS-T) and 3% hydrogen peroxide and blocked for 1 hr at room temperature in PBS-T containing 5% low-fat powdered milk. Membranes were cut according to the position of the expected molecular weight of the GR and α-tubulin, the latter used as an internal loading control. Incubations with primary antibodies were performed overnight at 4°C in PBS-T with 0.4 μg/ml rabbit polyclonal antibody raised against the GR alpha of mouse origin (GRm-20, Santa Cruz Biotechnology) and 4 μg/ml mouse monoclonal anti-α-tubulin antibody (Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa). Then, membranes were incubated with the corresponding secondary antibody coupled to horse radish peroxidase: 0.2 μg/ml goat anti-rabbit IgG (Chemicon International, Billerica, MA, USA) and 0.08 μg/ml goat anti-mouse IgG (Santa Cruz Biotechnology). Immunopositive bands were visualized by enhanced chemiluminescent detection using 1.25 mM luminol, 0.198 mM cumaric acid, 0.038 (v/v) hydrogen peroxide (100 vol.) in 100 mM Tris–HCl buffer, in a Fujifilm LAS-1000 chemiluminescent detection system. Densitometric analysis of corresponding bands was performed with the Image Gauge software (Fuji Photo Film Co. Ltd, Tokio, Japan). To account for loading differences, the intensity of the GR immunoreactive bands was normalized to the intensity of the α-tubulin immunoreactive band. In order to validate the semi-quantification method, the relationship between the amount of protein and optical density was analyzed. This ratio was linear in the range (50–100 μg of protein) used in this work.

A similar protocol was used for the detection of PCNA in the nuclear and postnuclear fractions of translocation experiment. Membranes were incubated overnight at 4°C with a mouse polyclonal PCNA antibody (Chemicon International) diluted with PBS-T at a concentration of 1 μg/ml. The secondary antibody was the goat anti-mouse previously described. Immunopositive bands were visualized by enhanced chemiluminescent detection as described above.

Testosterone Radioimmunoassay

Testosterone determination was performed as described by Pozzi and Ceballos (2000). Briefly, incubation media were analyzed without extraction. Depending on the treatment, steroids were assayed in 20–100 μl of incubation media. For the assay, serial dilutions of the standard curve, non-specific binding, maximum binding, and samples were incubated with 10,000 dpm of [^{3}H] testosterone (80.4 Ci/mmol, Perkin-Elmer, Boston, MA, USA). All the incubations were carried out in a final volume of 0.5 ml at 4°C for 18–22 hr. Testosterone antibody was from The Colorado State University (Co, USA) and was employed in a dilution of 1:125,000. The cross-reactivity of testosterone antibody with dihydrotestosterone was 15%. The buffer employed was 10 mM PBS, 1% gelatin, 20 mM EDTA, pH 7.4. Charcoal-dextran method was used to separate bound and free hormones. The sensitivity of the assay was 10 pg/ml. Mean intra-assay variation and inter-assay variation were 7.4% and 12.6%, respectively. Radioactivity in the supernatant (bound) was determined by liquid scintillation counting with a Wallac 1409 DSA equipment (Wallac Co, Turku, Finland). The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac Co). Androgen production was expressed as media contents per ml.

Statistical Analysis

Results were expressed as means of different assays ± standard error. Long-term in vitro experiments were analyzed with a randomized block design to minimize the variability between replicates due to the fact that there are variations in the amount of GR per mg tissue during the year. In this design, the blocks were the replicates carried out in different periods of the year and the treatments were the hormones and drugs tested. Data were checked for interaction by cell "means" plots. Lines corresponding to each replicate were roughly parallel (S-Figs. 3 and 5), meaning that there is no interaction between blocks and treatments (Quinn and Keough, 2002), that is, the replicates carried out in different periods of the year respond to the treatment in a similar manner. Therefore, the effect of the treatment is independent from the time of the year. Two-way ANOVAs followed by Tukey's multiple comparisons tests were used to detect significant differences among treatments and replicates.

GR protein from the treatment with melatonin and luzindole, and GR binding from the treatment with melatonin for 3 hr could not be analyzed with a randomized block design because the data did not meet the assumptions of the design. Data from the long-term incubations with GCs were unbalanced, thus they could not

be analyzed with a randomized block design. Cell means plot showed that the GR protein responded to the treatment in the same manner in all the replicates (S-Fig. 8). These experiments and seasonal variations in GR protein level were analyzed by one-way ANOVA followed by Tukey's multiple comparisons tests. Before statistical analysis, data were tested for normality and homoscedasticity by using Lilliefors and Bartlett's test, respectively. Total number of binding sites and GR protein levels were log transformed [$\ln_X$] to correct for heteroscedasticity. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK, USA). Differences were considered significant with $P < 0.05$. K_d was obtained by employing the Ligand Program (Ligand Software, David Rodbard, NIH).

RESULTS

GR Protein Isoforms in the Testis

The GRm-20 antibody recognized two immunoreactive bands of 55 and 82 kDa in the fresh homogenate (Fig. 1A, left panel). When the antibody was pre-incubated with the corresponding blocking peptide, none of the bands were visualized (Fig. 1A, right panel), suggesting that all of them correspond to specific GR immunoreactive bands. Western blot analysis of the cytoplasmic and plasma membrane-enriched fractions showed that both isoforms are localized in the cytoplasm and are not plasma membrane associated forms of the GR (Fig. 1B). The capacity of Dex to induce the translocation of the GR to the nucleus was used to assess the capacity of the isoforms to translocate to the nucleus. Figure 1C shows Dex-mediated GR nuclear translocation as the ratio between postnuclear and nuclear localization. The postnuclear/nuclear ratio of the 82 kDa band was significantly lower upon Dex exposure (two-way ANOVA, $F_{1,4} = 10.6$; $P = 0.03$), suggesting that this isoform translocate to the nucleus upon binding to the ligand. Conversely, the postnuclear/nuclear ratio of the 55 kDa band was not significantly different in the testes treated with Dex (two-way ANOVA, $F_{1,4} = 0.7$; $P = 0.3$), which suggests that this band is an alternative isoform of the GR, which do not translocate to the nucleus upon Dex exposure in the same

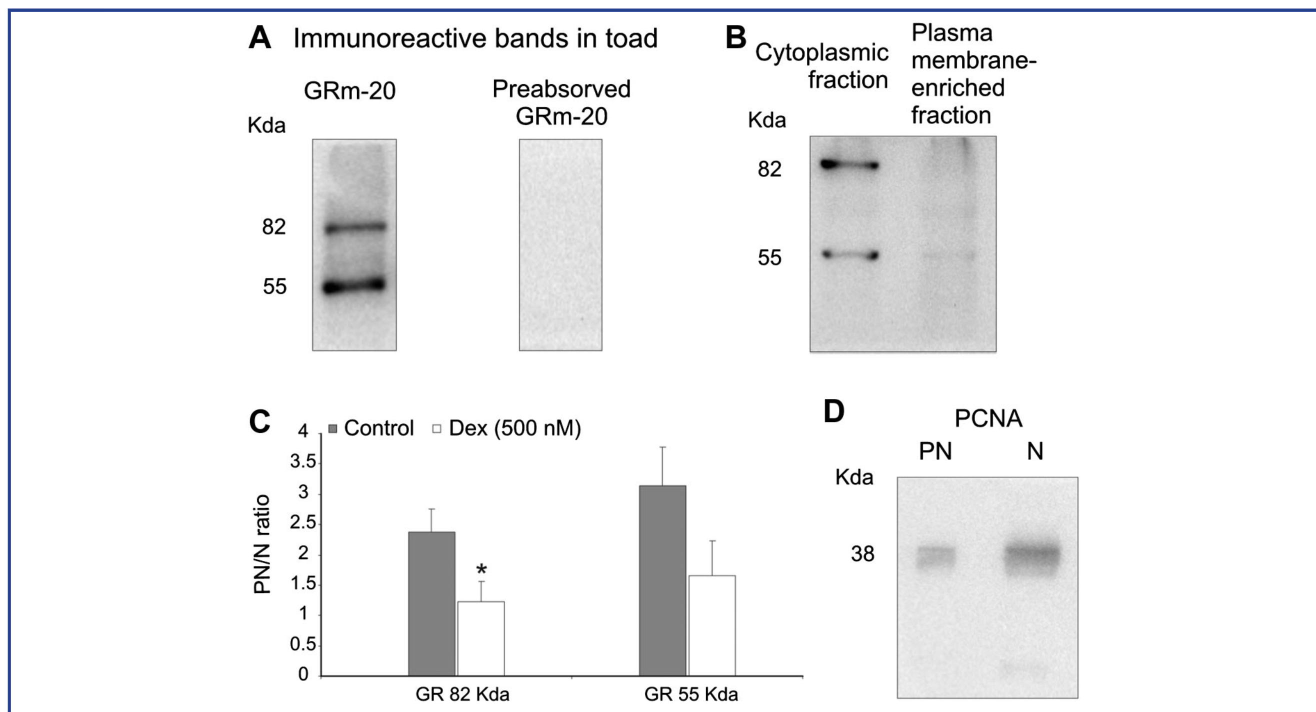

Figure 1. GR protein isoforms in toad testis. (A) Immunoreactive bands of 82 and 55 kDa detected after incubation of proteins from cell homogenate of toad testis with GRm-20 antibody (left panel), and with GRm-20 pre-incubated with blocking peptide (right panel). Apparent molecular weights were calculated with the Sigma gel software. (B) GR isoforms in cytoplasmic and plasma membrane-enriched fractions obtained by differential centrifugation. (C) Densitometric analysis of the translocation experiment. Toad testes were incubated with or without 500 nM Dex for 1 hr ($n = 5$). Nuclear (N) and postnuclear (PN) fractions were obtained by differential centrifugation and analyzed by Western blot. Results are expressed as the ratio between PN and N fraction. Data are expressed as the media $\pm$ standard error. Asterisk means significant differences between treatments with $P < 0.05$ for each isoform separately. (D) PCNA protein, a nuclear marker, detected by Western blot in the nuclear (N) and postnuclear (PN) fractions.

manner as the 82 kDa band does. Figure 1D shows that the separation of the nuclear and postnuclear fractions by differential centrifugation was performed correctly because the nuclear marker, PCNA, is more abundant in the nuclear fraction.

In *R. arenarum*, the characteristics of the 55 and 82 kDa isoforms are not known, therefore, both bands were considered separately in Western blot analysis. Omission of the primary antibody showed no detectable bands (data not shown).

Seasonal Changes in GR Proteins

Figure 2 shows that both GR bands exhibited seasonal variations. The band of 55 kDa exhibited the highest value during the postreproductive period (summer) and the lowest one during the pre-reproductive period (autumn–winter; one-way ANOVA, $F_{2,16} = 4.89$, $P = 0.02$). The band of 82 kDa had also the highest value during the postreproductive period but the lowest one during the pre-reproductive and the reproductive periods (winter–spring; one-way ANOVA, $F_{2,22} = 15.12$, $P < 0.001$). Seasonal variation in the sum of both bands was similar to the band of 55 kDa (one-way ANOVA, $F_{2,22} = 4.58$, $P = 0.02$).

Role of Androgens in the Regulation of the GR

Several in vitro approaches were carried out to evaluate the participation of androgens in the regulation of the GR. The analysis of the results showed that the replicates carried out in different periods of the year responded to the treatments in a similar manner (S-Fig. 3). Figure 3 shows that neither the addition of exogenous androgens, testosterone or DHT, nor the inhibition of the biosynthesis or the action of endogenous androgens modified the binding of the GR (two-way ANOVA, $F_{1,5} = 3.5$, $P = 0.1$; $F_{1,2} = 0.1$, $P = 0.8$; $F_{1,3} = 0.4$, $P = 0.6$; $F_{1,2} = 2.1$, $P = 0.2$, for the following treatments and its respective control: testosterone, DHT, CNK, and casodex). The effect of CNK on steroidogenesis was confirmed by the inhibition of testosterone production (two-way ANOVA, $F_{1,3} = 16.4$, $P = 0.03$ for control and CNK treatments; Fig. 3). Likewise, Figure 4A,B shows that none of the treatments modified the GR total protein content (for the 82 kDa band, two-way ANOVA, $F_{4,8} = 0.3$, $P = 0.9$ for control, testosterone, DHT, CNK, and casodex treatments; for the 55 kDa band, two-way ANOVA, $F_{4,8} = 0.99$, $P = 0.46$ for the same treatments). Taken together, these results suggest that in the testes of *R. arenarum* androgens do not seem to be involved in the regulation of the GR. The number of binding sites in control testes after 24 hr incubation was 6.17 ± 0.73 fmol/mg proteins while testosterone production by control testes was 8.92 ± 2.46 ng/ml.

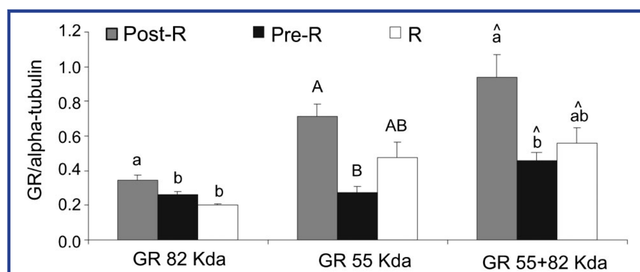

Figure 2. Seasonal pattern of GR protein measured by Western blot. Immunoreactive bands corresponding to 55 and 82 kDa were semi-quantified in cell homogenates from toads collected during the postreproductive (Post-R), pre-reproductive (Pre-R) and reproductive (R) periods. Values of GR protein were normalized to the corresponding alpha-tubulin level. Values are expressed as means of 6 (Post-R), 7 (Pre-R), and 12 (R) toads ± SE. Lowercase letters (82 kDa band), upper case letters (55 kDa band) and circumflex accent (82 + 55 kDa bands) were used to denote significant differences among periods for each immunoreactive band or for the sum of the 55 and 82 kDa bands. Different letters mean significant differences among periods with $P < 0.05$.

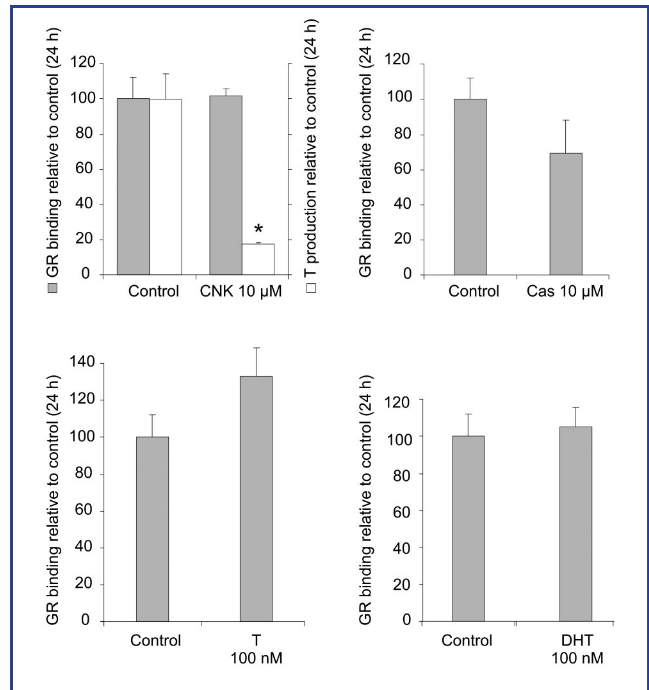

Figure 3. Effect of CNK, casodex, testosterone (T), and DHT on the GR number of binding sites. After incubation, testes were homogenized and postnuclear fraction was used in the binding assays ($n = 4$ for the treatment with CNK; $n = 6$ for the treatment with testosterone; $n = 3$ for the treatment with casodex, and $n = 3$ for the treatments with DHT). Results are expressed as number of binding sites relative to control at 24 hr. The effect of CNK on testosterone production is shown in white bars (top left). Values of testosterone synthesis in incubation medium were referred to the basal production after 24 hr incubation. All the results are expressed as means of the different experiments ± SE. Asterisk indicates significant differences among treatments with $P < 0.05$.

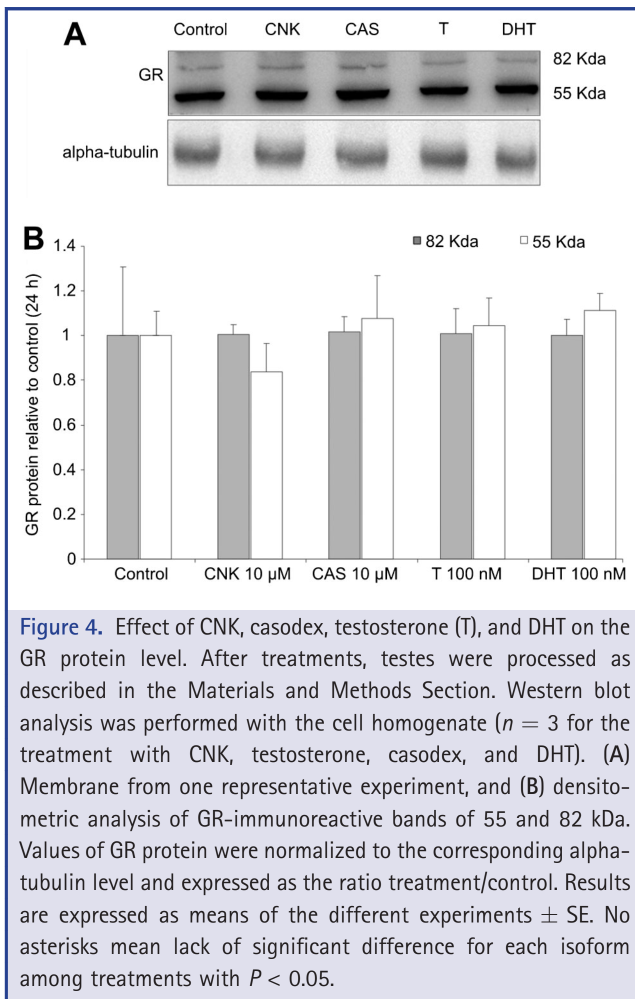

Figure 4. Effect of CNK, casodex, testosterone (T), and DHT on the GR protein level. After treatments, testes were processed as described in the Materials and Methods Section. Western blot analysis was performed with the cell homogenate ($n = 3$ for the treatment with CNK, testosterone, casodex, and DHT). (A) Membrane from one representative experiment, and (B) densitometric analysis of GR-immunoreactive bands of 55 and 82 kDa. Values of GR protein were normalized to the corresponding alpha-tubulin level and expressed as the ratio treatment/control. Results are expressed as means of the different experiments ± SE. No asterisks mean lack of significant difference for each isoform among treatments with $P < 0.05$.

Role of Melatonin in the Regulation of the GR

The effect of melatonin on the GR was determined by using two concentrations of melatonin in the presence or absence of the melatonin antagonist, luzindole. The analysis of the results showed that the replicates carried out in different periods of the year responded to the treatment in a similar manner (S-Fig. 5). As shown in Figure 5A, after a 24 hr treatment melatonin evoked a biphasic effect in the number of binding sites, that is, only the lowest concentration tested produced a significant decrease in this parameter (two-way ANOVA, $F_{2,10} = 8.4$, $P = 0.007$ for control, 40 pg/ml melatonin and 200 pg/ml melatonin treatments). The addition of luzindole completely abolished this inhibition, implying that the effect of the lowest concentration is mediated by a luzindole-sensitive receptor (Fig. 5B, two-way ANOVA, $F_{3,6} = 8.4$, $P = 0.8$ for control, luzindole-control, 40 pg/ml melatonin plus luzindole and 200 pg/ml melatonin plus luzindole). None of the concentrations of melatonin assayed produced significant differences in the number of binding sites after 3 hr treatment (Fig. 5C, one-way ANOVA, $F_{2,12} = 0.03$, $P = 0.97$ for control, 40 pg/ml melatonin and 200 pg/ml melatonin). The number of binding sites in control testes at 24 hr and 3 hr were 6.26 ± 0.88 and 15.85 ± 1.08 fmol/mg proteins, respectively.

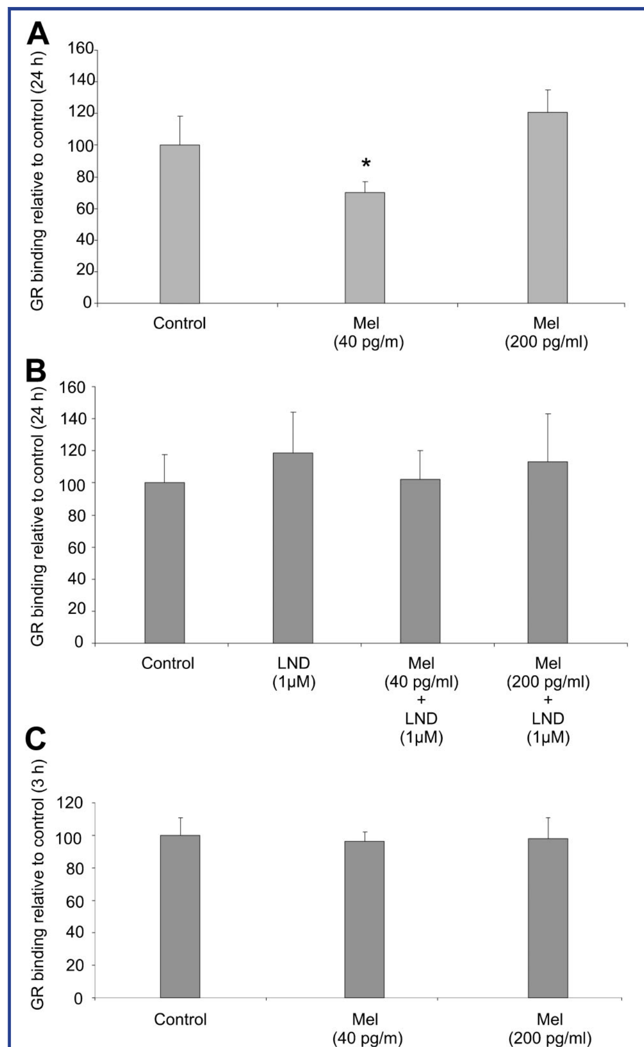

Figure 5. Effect of melatonin (Mel) and luzindole (LND) on GR number of binding sites. After treatments, testes were processed as described in the Materials and Methods Section. Binding assays were performed with the postnuclear fraction. (A) Number of binding sites relative to control at 24 hr from testes incubated with Mel ($n = 6$). (B) Number of binding sites relative to control at 24 hr from testes co-incubated with Mel and LND ($n = 3$). (C) Number of binding sites relative to control at 3 hr treatment with Mel ($n = 5$). Values are expressed as means of the different experiments ± SE. Asterisk indicates significant differences among treatments with $P < 0.05$.

When the effect of melatonin and luzindole on the testicular GR protein was evaluated, none of the concentrations of melatonin

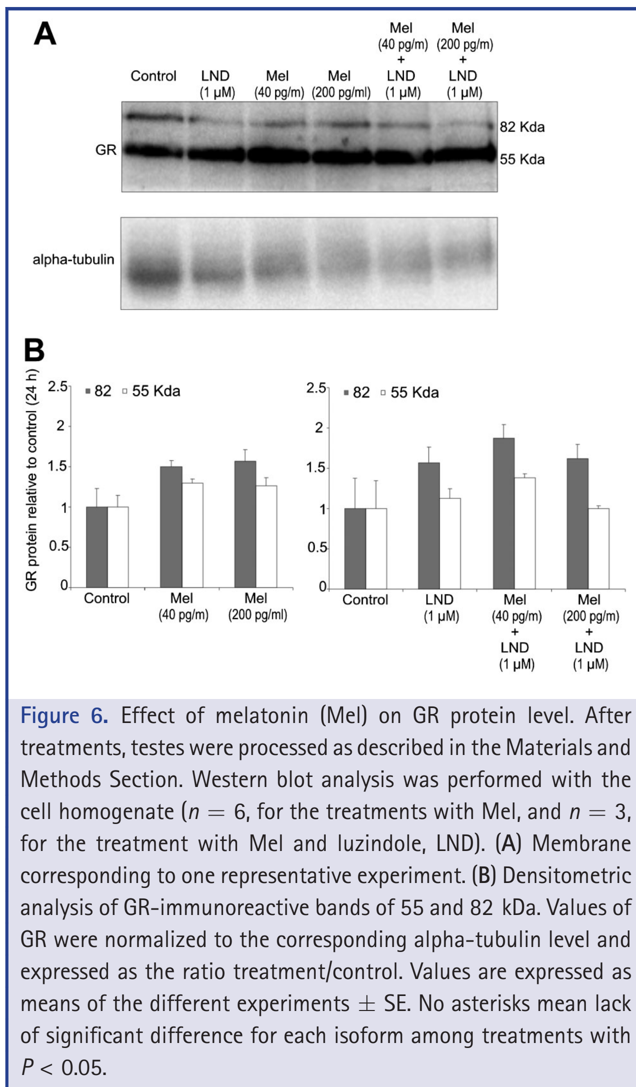

Figure 6. Effect of melatonin (Mel) on GR protein level. After treatments, testes were processed as described in the Materials and Methods Section. Western blot analysis was performed with the cell homogenate ($n = 6$, for the treatments with Mel, and $n = 3$, for the treatment with Mel and luzindole, LND). (A) Membrane corresponding to one representative experiment. (B) Densitometric analysis of GR-immunoreactive bands of 55 and 82 kDa. Values of GR were normalized to the corresponding alpha-tubulin level and expressed as the ratio treatment/control. Values are expressed as means of the different experiments ± SE. No asterisks mean lack of significant difference for each isoform among treatments with $P < 0.05$.

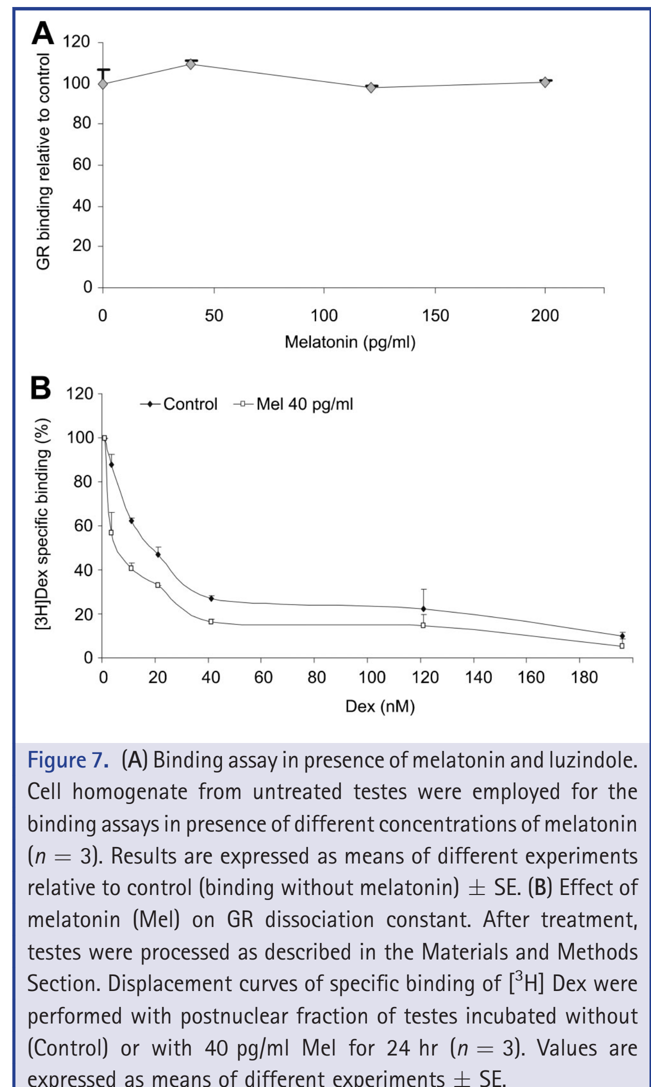

Figure 7. (A) Binding assay in presence of melatonin and luzindole. Cell homogenate from untreated testes were employed for the binding assays in presence of different concentrations of melatonin ($n = 3$). Results are expressed as means of different experiments relative to control (binding without melatonin) ± SE. (B) Effect of melatonin (Mel) on GR dissociation constant. After treatment, testes were processed as described in the Materials and Methods Section. Displacement curves of specific binding of [^{3}H] Dex were performed with postnuclear fraction of testes incubated without (Control) or with 40 pg/ml Mel for 24 hr ($n = 3$). Values are expressed as means of different experiments ± SE.

significantly modified the 82 or 55 kDa immunoreactive band (one-way ANOVA, $F_{2,15} = 0.82$; $P = 0.5$, for the 55 kDa band, and $F_{2,15} = 0.62$; $P = 0.5$, for the 82 kDa band; Fig. 6A,B). Luzindole also did not modify the GR protein (one-way ANOVA, $F_{3,8} = 0.74$; $P = 0.6$, for the 55 kDa band, and $F_{3,8} = 0.30$; $P = 0.83$, for the 82 kDa band; Fig. 6A,B).

The effect of the lowest concentration of melatonin after 24 hr treatment on the number of binding sites was not due to a direct interaction of melatonin with the GR because this parameter was not modified when the binding assay was performed with untreated testicular homogenate in the presence of melatonin (Fig. 7A).

To further analyze if the lowest concentration of melatonin modified the affinity of GR for Dex, competition experiments were performed with testicular postnuclear fraction after 24 hr incubation without (control) and with 40 pg/ml melatonin (Fig. 7B). The analysis of the data by the Ligand Programme showed no significant difference in K_d of melatonin treated testes (3.07 ± 1.22 nM) with respect to control (10.30 ± 2.69 nM). The slight difference in the displacement curve is due to the decrease in the number of binding sites and not to a modification in the affinity of the GR for Dex.

Role of GCs in the Regulation of the GR

The possibility that glucocorticoids auto-regulate their receptor in the testis was evaluated by in vitro incubation with Dex and RU486. The analysis of the results showed that the replicates carried out in different periods of the year responded to the treatment in a similar manner (S-Fig. 8). Figure 8 shows that only the highest concentration of Dex, 150 nM, which is equivalent to

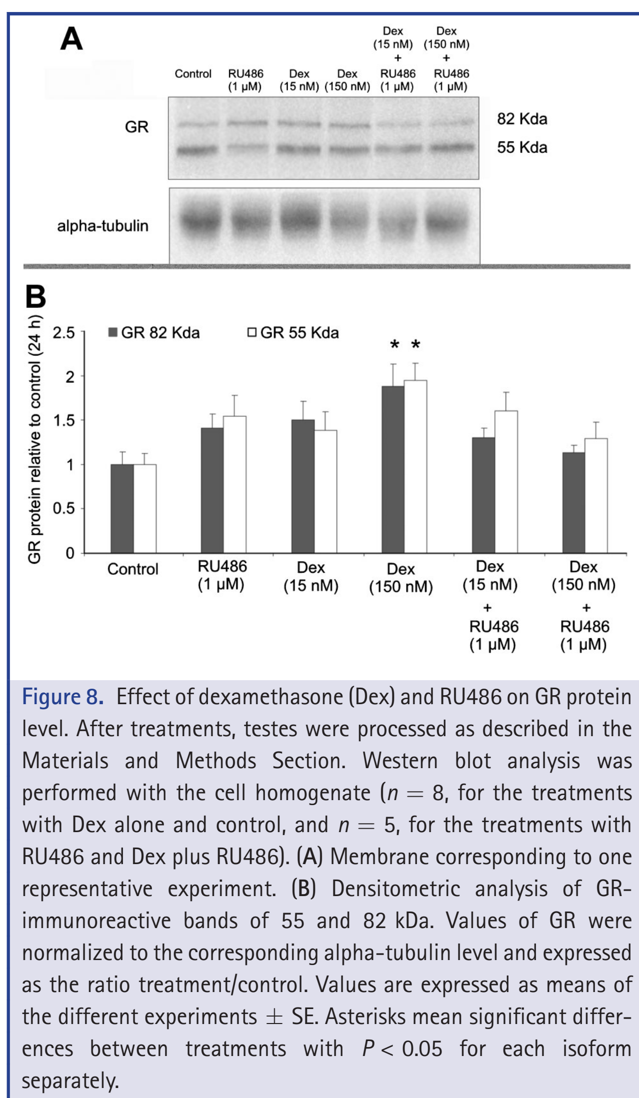

Figure 8. Effect of dexamethasone (Dex) and RU486 on GR protein level. After treatments, testes were processed as described in the Materials and Methods Section. Western blot analysis was performed with the cell homogenate ($n = 8$, for the treatments with Dex alone and control, and $n = 5$, for the treatments with RU486 and Dex plus RU486). (A) Membrane corresponding to one representative experiment. (B) Densitometric analysis of GR-immunoreactive bands of 55 and 82 kDa. Values of GR were normalized to the corresponding alpha-tubulin level and expressed as the ratio treatment/control. Values are expressed as means of the different experiments ± SE. Asterisks mean significant differences between treatments with $P < 0.05$ for each isoform separately.

the corticosterone concentration of the reproductive period, up-regulated both GR isoforms (one-way ANOVA, $F_{5,33} = 2.98$; $P = 0.02$, for the 55 kDa band, and $F_{5,33} = 2.75$; $P = 0.03$, for the 82 kDa band). The auto-regulation seems to be mediated by a receptor-mediated mechanism since the co-incubation with the GR antagonist, RU486, abolished the effect. Since incubation with RU486 alone did not alter the amount of GR protein in respect of control, it can be inferred that the increase of the protein produced by 150 nM Dex is not due to the stabilization of the receptor protein by binding to the ligand.

DISCUSSION

There are several aspects to take into account to determine the sensitivity of testicular cells to the action of GCs. Undoubtedly, plasma GC level is an important aspect, particularly in cold-blooded vertebrates having pronounced changes in this parameter. However, the amount of receptors for GCs and consequently the factors regulating these receptors are also important.

To start with the study of the amount of GR protein as well as the regulation of this receptor, it was necessary to validate the use of a heterologous antibody in the Western blot analysis. Preliminary results from Western blot suggested that the testis of *R. arenarum* expresses more than one immunoreactive form of the GR. Other authors have described various low molecular weight isoforms of the GR that fractionate into several bands upon electrophoresis (Yudt and Cidlowski, 2002; Oakley and Cidlowski, 2011). These isoforms correspond to alternative splicing or alternative translation initiation products, with uncertain function. The specificity of the GR-m20 antibody for toad GR was demonstrated by the pre-incubation of the antibody with the corresponding blocking peptide. Translocation experiments showed that the 82 kDa band significantly increases in the nuclear fraction upon Dex exposure, which suggests that this isoform translocates to the nucleus. On the contrary, the translocation of the 55 kDa band was not significant. The difference in the response to the Dex treatment between both bands could be due to a differential translocation mechanism, while the band of 82 kDa could represent the GRα isoform, the 55 kDa band could be equivalent to a splicing variant that translocate to the nucleus under different conditions (Yudt and Cidlowski, 2002). Since the GRm-20 antibody recognizes an epitope of 16–24 amino acids within the first 50 aminoacids of mouse GR, the 55 kDa isoform could not be a product of alternative translation initiation. This band was revealed in fresh as well as in 24 hr-incubated testes homogenates prepared in the presence of protease inhibitors, suggesting that the 55 kDa band is not a degradation product. This is not the first time that isoforms of GR are described by Western blot analysis; in ovine fetal hypothalamus and pituitary, immunoblotting analysis revealed two immunoreactive bands of 97 and 45 kDa (Saoud and Wood, '96). Particularly, employing the GRm-20 antibody several bands were detected in a cellular line of human hepatocytes (Psarra et al., 2005) but only a 90 kDa immunoreactive band was revealed in mouse thymocytes (Presman et al., 2006). These results clearly show that the number of immunoreactive bands could be organ and species specific. Nevertheless, in toad testis, the specific function of the several GR isoforms is unknown and has to be confirmed by analyzing the sequence(s) of the GR mRNA. For this reason, the effect of the hormones was evaluated separately on each isoform.

A seasonal study of the binding parameters of the testicular GR of *R. arenarum* was performed several years ago (Denari and Ceballos, 2006). In that study, it was demonstrated that in the toad there are significant differences in the apparent number of binding sites of the GR throughout the year (Table 1). However, it should be considered that the binding assay only measures unbound cytosolic GR and not the total amount of GR. Results in the present work showed that the total amount of the 55 kDa GR

Table 1. Annual pattern of the GR protein compared to the apparent number of binding sites.

Period	B_{app}	82 kDa GR protein	55 kDa GR protein
Post-R	339.18 ± 0.91^{â}	0.34 ± 0.03^{a}	0.71 ± 0.07^{A}
Pre-R	197.63 ± 23.91^{ê}	0.26 ± 0.02^{b}	0.27 ± 0.04^{B}
R	244.30 ± 24.15^{âê}	0.20 ± 0.01^{b}	0.48 ± 0.09^{AB}

Values of apparent number of binding sites (B_{app}) measured by Denari and Ceballos (2006) are expressed as fmol/mg protein. GR protein is expressed as the ratio of the semi-quantification of the GR protein/alpha-tubulin protein. Post-R: postreproductive period, Pre-R: pre-reproductive period, R: reproductive period. Different circumflex vowel letters mean significant differences in the B_{app} with $P < 0.05$. Different lower case letters mean significant differences in the 82 kDa GR protein with $P < 0.05$. Different upper case letters mean significant differences in the 55 kDa GR protein with $P < 0.05$.

isoform and the sum of the amount of both isoforms have the same annual pattern that the apparent number of binding sites reported by Denari and Ceballos (2006; Table 1). Instead, the annual pattern of the 82 kDa isoform does no coincide with the annual pattern of the number of binding sites (Table 1). Surprisingly, the binding kinetics studied by Denari and Ceballos (2006) fitted to a one-site model, suggesting that both isoforms bind to Dex with the same affinity. The parallelism between the annual pattern of binding sites and the 55 kDa isoform could be due to the fact that the 55 kDa is the predominant band in the testis, specially during the reproductive period (Table 1 and Fig. 2). The lowest amount of receptors measured either by Western blot or by binding assay is found during the pre-reproductive season (winter), a season characterized by high plasma levels of testosterone and DHT and low concentrations of corticosterone (Canosa et al., 2003; Denari and Ceballos, 2005). As in the testis of *R. arenarum*, seasonal changes in the number of cytosolic corticosteroid receptors were found in the brain of the House Sparrow, the number of corticosteroid receptors being also lower during the winter (Breuner and Orchinik, 2001). Therefore, seasonal regulation of the GR could modulate the magnitude of the effect of GCs in different organs and species, including the testis of *R. arenarum*.

Taking these results into account, the next question was whether the high concentration of androgens and low concentration of GCs found in *R. arenarum* during the pre-reproductive season and/or the low and constant concentration of melatonin found in other amphibians during the winter could regulate the GR during the pre-reproductive period. Our results suggest that in *R. arenarum* androgens are not involved in the regulation of GR. Several approaches, including the inhibition of androgen biosynthesis, the use of an androgen receptors antagonist, and also the treatments with exogenous testosterone or 5α-DHT in physiological concentrations, support this conclusion. Conversely, GCs seem to autoinduce the levels of the GR protein in the testis. A high concentration of Dex, 150 nM, which is equivalent to the concentration found during the reproductive period, up-regulate both, the 55 and 82 kDa GR isoforms, in the same manner. Extrapolating from these in vitro results, low plasma concentration of GCs during the pre-reproductive period could be maintaining low levels of GR in the testis, when androgens synthesis is maximal. However, it should be considered that as GCs seem to regulate both GR isoforms in the same manner, other factors must be producing the differences between the annual pattern of the 55 and 82 kDa isoforms.

As mentioned in the Introduction, melatonin is another hormone expressing seasonal variations that could modify the sensitivity of the testes to the action of GCs. The in vitro treatment with melatonin concentrations in the order of 10^{-9} M produced a biphasic effect on the GR total number of binding sites, with a decrease with the lower concentration and no change with the higher one. Furthermore, when testes were incubated with melatonin and a membrane melatonin-receptor antagonist, luzindole, the effect of the lowest concentration of melatonin disappeared while the lack of effect of the highest one remained constant. These results indicate that a low concentration of melatonin modifies the binding of the GR in toad testis by a membrane receptor-mediated mechanism. It is well known that melatonin plays a key role in the regulation of circadian rhythms and reproduction in several species (Morgan et al., '94; Mayer et al., '97). Particularly, in females of *R. arenarum*, both pineal organ extract and melatonin directly inhibited in vitro ovulation (de Atenor et al., '94). In addition, several in vitro effects of physiological concentrations of melatonin have been described in germ line, Sertoli and Leydig cells in the testis of *Rana esculenta* (d'Istria et al., 2003, 2004).

One question that remains to be answered is why the lower concentration of melatonin evokes a membrane receptor-dependent inhibition in the number of binding sites whereas the higher one has no significant effect on the same parameter. Since several types of melatonin receptors have been described in diverse species and tissues (von Gall et al., 2002), the lack of effect of 200 pg/ml may be due to the interaction of the hormone with at least two different receptors, probably with antagonistic effects. Supporting this idea, two melatonin receptors, Mel 1a (MT1) and Mel 1c, were described in chicken splenocytes, and they act through different second messengers that produce opposite effects

on cellular proliferation (Markowska et al., 2004). Regarding amphibians, three melatonin receptors MT1 (Mel 1a), MT2 (Mel 1b), and Mel 1c have been described in *X. laevis* tectal cells (Prada et al., 2005), and recently the presence of one type of melatonin receptor (Mel 1b) transcript has been isolated from testis of *Pelophylax esculentus* (Serino et al., 2011). These authors suggest that the presence of a melatonin receptor transcript in the frog testis corroborates their previous results obtained in in vitro experiments, which suggest that melatonin could act directly in male frog gonads (Izzo et al., 2004). The fact that the effect of 40 pg/ml of melatonin is inhibited by luzindole allows also the conclusion that at least one type of melatonin receptor is expressed in the testis of *R. arenarum*. On the other hand, the lack of effect of 200 pg/ml melatonin on the number of binding sites after a 24 hr treatment could be due to the interaction of melatonin with an orphan nuclear receptor or with cytosolic calmodulin (Ning and Sánchez, '96; Reiter et al., 2010). In this regard, at the highest concentration, melatonin could be interacting with two types of receptors or with calmodulin, producing antagonistic effects on the GR complex that result in the biphasic effect. Surprisingly, none of the concentrations of melatonin modified the 82 or the 55 kDa bands, suggesting that the effect of this hormone on the receptor binding capacity is not related to the amount of GR protein. One possible explanation for the effect of the lowest concentration of melatonin after 24 hr incubation is that this hormone modifies the binding properties of the GR. However, this effect is not mediated by an interaction between melatonin and the GR complex, since the number of binding sites was not changed when the binding assay was performed with untreated fresh homogenate in the presence of melatonin (Fig. 7A). Considering that the GR has a phosphorylation consensus motif that can be recognized by MAPKs, modulating its function (Ismaili and Garabedian, 2004), and that melatonin regulates the function of the MAPKs, JNK, and p38 (Joo and Yoo, 2009), we speculated that in toad testis melatonin modulates the GR by modifying the phosphorylation status of the GR complex. If this was true, short-term incubation (3 hr) of testes with melatonin would modify the binding capacity of the GR. However, after short-term treatment experiments, none of the concentrations assayed produced significant differences in the number of binding sites, suggesting that melatonin does not modify the number of binding sites by affecting the phosphorylation status of the GR complex. As mention under Introduction, GR complex is composed by heat shock proteins (hsp), being the most conspicuous one the hsp90. Thus, it is possible to hypothesize that the incubation with melatonin for 24 hr could alter the association of the receptor with this chaperone, a fundamental component for the interaction with GCs. This effect was observed in rat liver and kidney, in which in vivo treatments with high doses of melatonin (0.9%, w/v) produced sex-specific changes in the GR binding parameters B_{max} and K_d, these changes being associated with alterations in the interaction of the receptor with hsp70 and hsp90 (Elakovic et al., 2009). However, this hypothesis needs to be tested in toads by analyzing the level of hsp associated with the receptor in testes treated with and without melatonin.

In conclusion, two cytoplasmic isoforms of the GR with unknown function seems to be present in the testis of the anuran *R. arenarum*. Regarding its regulation, the highest concentration of circulating GCs seems to autoinduce the expression of both GR protein isoforms. On the contrary, a low physiological concentration of melatonin (40 pg/ml) produces a decrease in the number of binding sites after a 24 hr treatment by a receptor mediated mechanism. This decrease is obtained with a concentration of melatonin that has been described in *R. perezi* as corresponding to cold days of winter, when melatonin rhythm disappears (Delgado and Vivien-Roels, '89; Delgado et al., '93). Therefore, it is probable that melatonin down-regulates the GR binding in toad testes during the winter when a decrease in the apparent number of binding sites for GC (Denari and Ceballos, 2006) is described. Furthermore, this down-regulation of the GR could be involved in decreasing the sensitivity of the testis to the GC action during the pre-reproductive season, a period characterized by a high concentration of plasma androgens and a low amount of GC. On the contrary, melatonin does not seem to be involved in the decrease of the GR protein. More studies are required to determine the mechanism by which melatonin modifies the number of binding sites.

ACKNOWLEDGMENTS

The monoclonal antibody anti-α-tubulin developed by Joseph Frankel and E. Marlo Nelsen was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. We thank Dr. J. C. Calvo for his assistance with the Ligand Program. This work was supported by grants from Universidad de Buenos Aires (UBACYT X042), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2433) and Agencia Nacional de Promoción Científica y Tecnológica de la Argentina (PICT 32219) to NRC.

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