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The expression level of frog relaxin mRNA (*fRLX*), in the testis of *Rana esculenta*, is influenced by testosterone

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Summary

Frog relaxin (*fRLX*) belongs to the relaxin/insulin gene family present in the testis of *Rana esculenta* and is specifically expressed by Leydig cells. Since the expression of *fRLX* transcript changes during the reproductive cycle and is more abundant when circulating levels of androgens are relatively high, we investigated the effect(s) of testosterone and its antagonist (cyproterone acetate, CPA) on its expression pattern, in the testis of the frog *Rana esculenta*. Results from *in vivo* and *in vitro* experiments demonstrate that testosterone strongly induces a significant increase of *fRLX* mRNA expression in frog testes and, this effect is counteracted by CPA, supporting the existence of intratesticular (autocrine/paracrine) mechanisms of action. Interestingly, in both the control and testosterone-treated testes, *fRLX* mRNA expression was markedly decreased 24 h post-treatment, as compared to that measured at 2 h and 8 h post-treatment, suggesting that factor(s), other than testosterone, may act(s) in controlling its expression. In addition, RT–PCR analysis and *in situ* hybridization performed on frog testis injected with CPA for 15 days, on alternate days, showed a strong decrease of *fRLX* expression, suggesting that CPA counteracts the effect of testosterone on *fRLX* expression. Taken together our results strongly indicate that changes in the production, by the Leydig cells, of both testosterone and *fRLX* may represent a marker for the study of Leydig cell activity in the testis of the frog *Rana esculenta*.

Key words: relaxin, testosterone, cyproterone acetate, gene expression, *Rana esculenta*.

Introduction

Relaxin (RLX) and relaxin-like factor (RLF) are small peptide hormones belonging to the insulin superfamily which are secreted in both mammalian sexes (Sherwood, 1994). All the proteins of this family share similar tertiary structure, in fact, the pro-prehormone peptide consists of a signal peptide, the common B-C-A heteromeric structural organization in which the B and A domain are covalently linked by two intradomain disulfide bonds. In the B domain the highly conserved R-XXX-R relaxin receptor binding motif is present (Bryant-Greenwood and Schwabe, 1994); a post transcriptional modification produces the removal of the signal peptide and of the C domain (Steiner, 1998).

In mammals, RLX is mainly secreted by the corpus luteum, a glandular structure in the ovary, and is generally associated with the physiology of the female reproductive tract (Sherwood, 1994) having a well-recognized role in parturition (Bryant-Greenwood and Schwabe, 1994). Nevertheless, RLX is responsible for a wide range of functions, with responsive tissues in the brain, heart, kidney and skin, besides the more classical organs of the female reproductive system (Bathgate et al., 2003).

By contrast, RLF is highly expressed in adult Leydig cells and at lower levels in the theca cells of the corpus luteum, the trophoblast, breast, and a variety of other tissues (Tashima et al., 1994; Pusch et al., 1996; Bathgate et al., 1996; Balvers et al., 1998; Spiess et al., 1999). *RLF* knockout mice were cryptorchid suggesting that RLF is involved in the gubernaculum formation necessary for the testis descent in the scrotum (Zimmermann et al., 1999; Nef and Parada, 1999). Recently, it has been demonstrated that, in the testis, besides its action on the gubernaculums, RLX suppresses apoptosis and has a paracrine action as survival factor for male germ cells (Kawamura et al., 2004).

Frog relaxin (*fRLX*) is the first form of RLX molecularly characterized in the testis of a non mammalian vertebrate (De Rienzo et al., 2001). It is highly expressed by the interstitial Leydig cells and the transcript levels change during the annual reproductive cycle, suggesting its involvement in spermatogenesis. Interestingly, although *f*RLX has a RLX

structure, its cellular localization is similar to that of the RLF. In addition, phylogenetic analysis suggests that *f*RLX sequence can represent an ancestral form of relaxin from which both modern mammalian relaxin and RLF might have evolved, for female and male functions, respectively (De Rienzo et al., 2001).

To date, the control of the expression of the *RLX* and *RLF* genes is still unclear; the corpus luteum produces a small but consistent rise in plasma RLX after the LH surge (Stewart et al., 1990). However, the transcription of *RLF* is mediated by steroidogenic factor I (Zimmermann et al., 1998) and it seems to be influenced by testosterone (Paust et al., 2002; Ivell et al., 2003).

Since *fRLX* transcript is more abundant when circulating levels of androgens are relatively high and is differently expressed during the frog reproductive cycle (De Rienzo et al., 2001), investigated the effect(s) of the administration of testosterone and its antagonist (cyproterone acetate; CPA) on *fRLX* mRNA expression in the testis of the frog, *Rana esculenta*.

Material and methods

Animals

Adult male frogs, *Rana esculenta* L., were supplied monthly from the surroundings of Naples by a local dealer. Animals were maintained in plastic tanks (23×16×11 cm) with food and water available *ad libitum*. The animals were killed by decapitation under anesthesia (MS-222; Sigma Chemical Co., St Louis, MO, USA) and the testes were dissected and quickly frozen by immersion in liquid nitrogen and stored at –80°C until RNA extraction.

This project was approved by the Italian Ministry of Education and Research (MIUR).

In vivo testosterone treatment

In May (when testosterone levels are low) adult frogs (N=35) were divided into three groups as follows: five frogs, used as initial control, were immediately killed; 15 animals were treated with a single injection of 0.2 mg of testosterone (T) in 100 μl of Krebs-Ringer solution at pH 7.4 (KRB); 15 animals were treated with a single injection of 0.2 mg of T plus 2 mg of cyproterone acetate (CPA) in 100 μl of KRB. The animals (5 animals/group/sample time) were sacrificed at different time points: 2, 8 and 24 h after the injection.

In vitro testosterone treatment

In May adult frogs (N=36) were killed and the testes were placed in tubes (24 testes/tubes) containing: (1) KRB alone; (2) KRB plus T (10^{-6} mol l^{-1}) and (3) KRB plus T (10^{-6} mol l^{-1}) and CPA (10^{-5} mol l^{-1}), for different times (2, 8 and 24 h). At the end of the treatment the testes were removed from the solution and frozen for RNA extraction. In addition, the testes excised from four frogs were immediately frozen for extraction of RNA and used as initial controls.

In vivo CPA treatment

In March (when testosterone levels are high) adult frogs (N=25) were divided into three groups as follows: five frogs, used as initial control, were immediately killed; 10 animals were injected on alternate days with KRB alone; 10 animals were injected on alternate days with a dose of CPA (0.33 mg 100 μl^{-1} KRB). The CPA- and KRB-injected frogs were killed 15 days after the first injection and the testes were removed and frozen for RNA extraction. In addition, three testes/group were fixed in Bouin's fluid, and processed for *in situ* hybridization.

Preparation of total RNA and northern blot analysis

Total RNA from the testes of the frog *Rana esculenta* were prepared with the procedure described (Sprenger et al., 1995).

Total RNA (20 μg for each sample) was fractionated by electrophoresis on a 1% agarose gel containing 2.2 mol l^{-1} formaldehyde and then transferred to a nitrocellulose membrane by overnight capillary blotting (Hybond^{TM}-N+; Amersham Pharmacia Biotech, Bucks, UK). The filters were prehybridized for 5–6 h at 65°C in 5× SSC, 5× Denhardt's, 100 μg ml^{-1} salmon sperm DNA and 50 mmol l^{-1} sodium phosphate at pH 7.0 and hybridized with a ^{32}P-labelled probe (2×10^6 c.p.m. ml^{-1}) corresponding to the *fRLX* cDNA (De Rienzo et al., 2001) at 65°C overnight. The filters were washed twice for 30 min at 65°C in 0.2× SSC and 0.1% SDS and were then exposed to X-ray film (HR-H, FUJI). In addition, the same filters were stripped in 0.1× SSC and 0.1% SDS and rehybridized with a *fP1*cDNA probe as a positive control with the same conditions as above.

RT–PCR

The levels of *fRLX* mRNA were determined by reverse transcription-polymerase chain reaction technique (RT–PCR) using as control the levels of *fP1* mRNA.

First-strand cDNA was synthesized using 5 μg of total RNA from control and CPA-treated testes, 500 ng oligo$(dT)_{18}$ primer (Promega, Heidelberg, Germany), 0.01 mol l^{-1} DDT and 200 i.u. Superscript II RT enzyme (Life Technologies, Paisley, UK) in a total volume of 20 μl according to the manufacturer's instructions (Life Technologies, Paisley, UK). Portions (1.5 μl) of the resultant cDNA were used to amplify, by PCR, a 265 bp fragment of *fRLX* (EMBL accession number: AJ298874) containing 211 bp of coding region and 54 bp of 3′ UTR and a 356 bp fragment of *fP1* (a *Rana esculenta* mRNA for acidic ribosomal protein 1; accession number: AJ298875). The reaction was performed in 1.5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris–HCl pH 9.0, 50 mmol l^{-1} KCl and 0.1% Triton X-100, using 1 i.u. *Taq* DNA polymerase (Promega, Heidelberg, Germany) and 5 pmol oligonucleotide primers (*fRLX* forward primer: 5′-tgtatgcagagagccacat-3′, *fRLX* reverse primer: 5′-gagtgcttgctctgcagac-3′, *fP1* forward primer: 5′-tggctggacagc-taacatg-3′, *fP1* reverse primer: 5′-tcaggacatcacatactggc-3′). Amplifications, carried out for 25 cycles, were as follows: 94°C for 40 s, 56°C for 40 s and 72°C for 40 s. PCR products were separated on 1.5% agarose gel in 1× TAE buffer using ethidium bromide for visualization. The quantification of the

bands was carried out with a Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). The PCR products were sequenced to verify the specificity of the amplification.

In situ hybridization

The DIG-11-UTP-labelled RNA probe used for in situ hybridization experiments on semithin sections corresponds to the fRLX cDNA insert. The *in situ* hybridization on control and CPA-treated testes was performed as reported previously (De Rienzo et al., 2001).

Results

In vivo testosterone treatment

To investigate if testosterone (T), the sexual hormone, controls *fRLX* mRNA expression, an *in vivo* experiment was performed. Frogs were injected with T alone or in combination with its antagonist cyproterone acetate (CPA). Total RNA was extracted from the hormone-treated frog testes after 2, 8 and 24 h of treatment. RT–PCR analysis was performed to detect *fRLX* expression using, as a control, the expression of *fP1* (Fig. 1A,B).

A strong increase of *fRLX* mRNA expression was found in the testes of testosterone-treated frogs in all three groups as compared to that found in the testes of the control (Fig. 1A,C). By contrast, no differences of *fRLX* expression were found between the T plus CPA and the control testes (Fig. 1A,C).

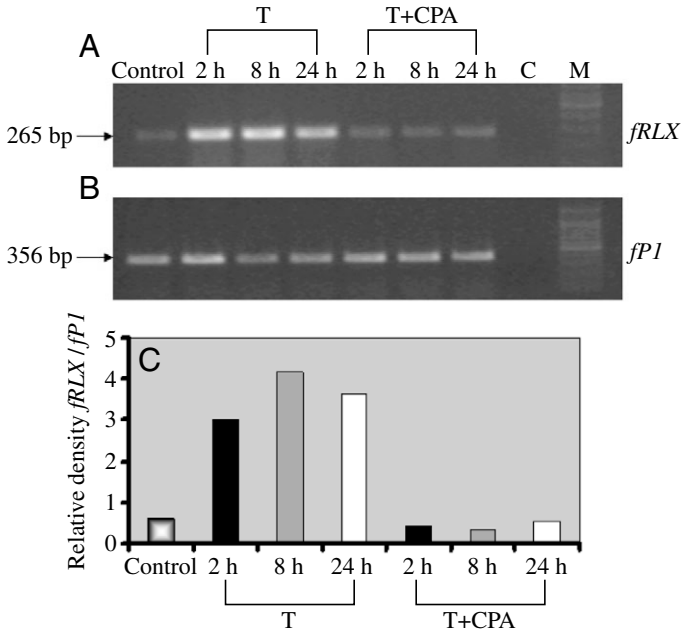

Fig. 1. Agarose gel electrophoresis of RT–PCR products. (A) *fRLX* mRNA expression on frog testis treated with T and T in combination with CPA at 2, 8 and 24 h. (B) *fP1* expression on the same samples used as control. Control, initial control frog testis. Lane C, control PCR without cDNA; M, molecular mass marker VIX (Roche Diagnostic). (C) Relative density of the *fRLX* and *fP1* band measured with Gel Doc.

In vitro testosterone treatment

To better understand the effect of testosterone treatment an *in vitro* experiment was performed. The *fRLX* expression was evaluated by northern blot analysis on total RNA extracted at different time from frog testis incubated with vehicle alone, T, and T in combination with CPA using *fRLX* cDNA as a specific probe (Fig. 2A). The same filter was rehybridized using *fP1* cDNA (De Rienzo et al., 2001) as control for RNA loading (Fig. 2B). The single band observed corresponds to a transcript of about 1.6 kb in length, which is the size of the isolated cDNA clone (De Rienzo et al., 2001).

No differences in *fRLX* mRNA expression were found in the testes incubated in vehicle alone at 2 and 8 h as compared with the control (Fig. 2A,C), whereas a drastically decrease was observed after 24 h of treatment (Fig. 2A).

By contrast, a strong increase in *fRLX* mRNA expression was found in the testes incubated in vehicle containing T at 2 and 8 h of treatment, whereas in T-incubated testes at 24 h *fRLX* expression showed a decrease, as compared to the control. Worthy of note is that the level of *fRLX* expression found after 24 h of testosterone treatment was higher than that observed in testes incubated in vehicle alone at the same time (Fig. 2A,C).

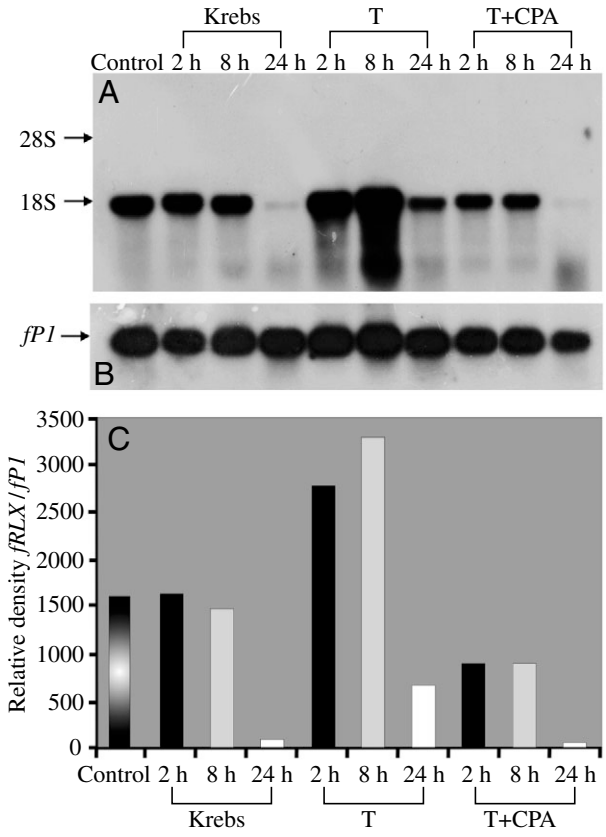

Fig. 2. (A) Northern blot analysis of total RNA extracted from frog testis hybridized with the *fRLX* cDNA as probe. Each lane contained 20 μg of total RNAs extracted from testis incubated with vehicle alone (Krebs), testosterone (T) and T in combination with CPA. (B) The same filter was hybridized with the *fP1* cDNA probe as control. (C) Relative density of the *fRLX* and *fP1* band measured with Gel Doc.

fRLX mRNA expression in testosterone plus CPA-treated testes at 2 and 8 h was lower than the control and the vehicle-treated testes at any time of incubation (Fig. 2A,C), whereas no differences were found between 24 h vehicle and T+CPA-treated testes (Fig. 2A,C).

In vivo CPA treatment and spatial localization of fRLX mRNA

To evaluate the testosterone control on the *fRLX* transcription an *in vivo* CPA treatment was performed. The frogs were treated with CPA for 15 days and the *fRLX* transcription levels were determined by RT–PCR. As reported in Fig. 3A CPA drastically reduced the *fRLX* mRNA expression at 15 days. No differences in *fRLX* expression were detected at 15 days in the control testes as compared to that of the initial control testis (Fig. 3A).

To ascertain the decrease in *fRLX* expression CPA-treated frogs at 15 days, *in situ* hybridization experiments were performed on control and treated frog testis. A strong signal was detected in the interstitial tissue around the germinal compartment (De Rienzo et al., 2001) in control testes (Fig. 3C), whereas the levels of *fRLX* transcript were visibly reduced in the 15-day CPA-treated testes (Fig. 3D).

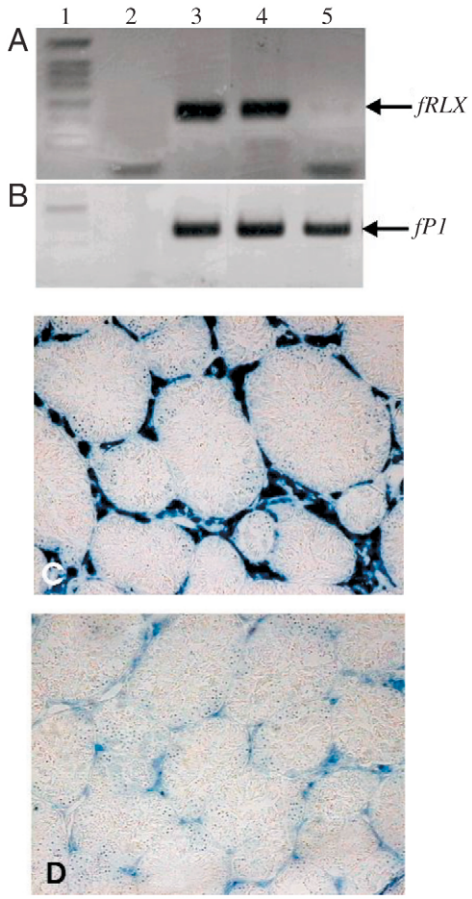

Fig. 3. *In vivo* CPA treatment. (A) Levels of *fRLX* mRNA determined by RT–PCR on initial control (lane 3), 15 days control (lane 4) and 15 days CPA-treated (lane 5) frog testis. Lane 1: molecular mass marker VIX (Roche Diagnostic); lane 2: control PCR. (B) *f*P1 mRNA levels used as control. (C,D) *In situ* hybridization of *fRLX* antisense mRNA on the control (C) and in the CPA-treated frog testis (D).

Discussion

The functional role of relaxin/insulin proteins in the mammalian testis is not fully understood, although, studies in knockout mice indicated that the relaxin-like factor (RLF) influences the gubernaculums to induce, at birth, the descent of the testis into the scrotum (Zimmerman et al., 1999; Nef and Parada, 1999). It should be noted that in lower vertebrates testicular descent does not occur and spermatogenesis is susceptible to seasonal thermal changes (Rastogi and Iela, 1992). In this context, frog relaxin (*fRLX*), a member of the relaxin/insulin gene family, has been found in the testis of *Rana esculenta*, and it is specifically expressed by Leydig cells (De Rienzo et al., 2001). Interestingly, the expression of its transcript changes throughout the frog annual cycle (De Rienzo et al., 2001) strongly suggesting that it might contribute to the efficiency of spermatogenesis in these seasonal breeders, possessing a reproductive cycle regulated by endocrine and environmental factors and a cystic organization of the testis that favours spermatogenesis (Rastogi et al., 1978; Rastogi and Iela, 1992). In fact, *fRLX* transcript is more abundant when circulating levels of androgens are relatively high and its expression pattern correlates well with the frog steroidogenic and spermatogenetic wave (De Rienzo et al., 2001).

It is worth remembering that, in the frog testis, androgens are produced by the Leydig cells and are necessary for spermatogonial proliferation and spermatid formation (Rastogi and Iela, 1992; Minucci et al., 1992). Therefore, it is possible to hypothesize the existence of a relationship between androgen production, *RLX* expression and spermatogenesis.

In an attempt to obtain more information about the influences of androgens on *fRLX* mRNA expression, if any, we evaluated the effect of testosterone and its antagonist (cyproterone acetate; CPA), by *in vivo* and *in vitro* experiments, in the testis of the frog *Rana esculenta*. Experiments were performed in two different periods of the frog reproductive cycle: May, when testicular testosterone and *fRLX* mRNA levels are low, and March, when testicular testosterone and *fRLX* mRNA are at their highest levels (De Rienzo et al., 2001). It is relevant to note that, in the testis of animals injected in May, testosterone treatment strongly induced a significant increase in *fRLX* expression, at all times post-injection, and this effect was counteracted by CPA, supporting the existence of intratesticular (autocrine/paracrine) mechanisms of action (Fig. 1). Our data are also supported by the observation that *fRLX* expression increases during the frog annual cycle in concomitance with the highest testosterone concentration (De Rienzo et al., 2001).

In addition, pieces of testis in May, incubated with testosterone, show a significant increase in *fRLX* expression at 2 and 8 h, with these effect counteracted by CPA (Fig. 2A). Interestingly, *fRLX* expression strongly decreased in the testis of all the groups at 24 h of incubation, suggesting that factor(s)

other than testosterone may act(s) in controlling its expression. Moreover, it has also been suggested that *fRLX* expression could be under hypophysal control, in fact, *f*RLX transcript has been detected at a low level in the interstitial compartment of hypophysectomized frogs 30 days after surgical depletion (De Rienzo et al., 2001). In mammals, other studies reported that LH/hCG is essential to induce relaxin-like factor (RLF) expression in the adult Leydig cells of the mouse (Balvers et al., 1998). However, a pituitary control of RLF has been indicated, using hypogonadic mice lacking an active pituitary–gonadal axis caused by a deletion in the hypothalamically expressed gene for GnRH, with consequent gonadotropin deficiency (Scott et al., 1990; Cattenach et al., 1997; Charlton et al., 1983). Here, *RLF* expression is totally absent, suggesting that Leydig cells seem to be arrested in a prepubertal state of differentiation (Balvers et al., 1998).

In addition, RT–PCR analysis performed on the testes of frogs in March (when testosterone levels are high), injected for 15 days with CPA, showed a strong decrease in *fRLX* expression (Fig. 3A) and this data was also confirmed by *in situ* hybridization. In fact, we observed a reduction of the hybridization signal from the interstitial Leydig cells, suggesting that CPA counteracts the effect(s) of endogenous testosterone on *fRLX* expression (Fig. 3C,D).

Furthermore, since GnRH and its long acting agonist, buserelin (GnRHa), directly stimulate androgen production and spermatogonial multiplication in the frog testis (Pierantoni et al., 1984a; Pierantoni et al., 1984b; Minucci et al., 1986; Fasano et al., 1990; Rastogi et al., 1990), we performed experiments in which testis were incubated with GnRHa in order to stimulate testosterone secretion *in vitro*. Interestingly, *fRLX* expression progressively increases in testes incubated with GnRHa and reaches a peak at 6 h (G. De Rienzo, G. Izzo, D. Ferrara and S. Minucci, unpublished data), confirming the influence of testosterone on its expression. Taken together, our results indicate that testosterone probably acts directly in controlling *fRLX* expression in the frog testis. In this context, consideration should be given to our recent data which emphasize the fact that melatonin interferes with Leydig cell activity, thereby inhibiting the GnRH-induced testosterone secretion *in vitro* (d'Istria et al., 2004) and induces the complete disappearance of *fRLX* transcript from the testis of frogs injected with melatonin. This supports the hypothesis that this hormone exerts an inhibitory effect on Leydig cells by modifying their functional state.

In conclusion, our present study shows that *fRLX* expression, in the testis of the frog *Rana esculenta*, is regulated by testosterone both *in vivo* and *in vitro*, and this effect is counteracted by CPA. Lastly, *fRLX* together with testosterone may be considered a marker for the study of Leydig cells activity, in the testis of the frog *Rana esculenta*.

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