General and Comparative Endocrinology 187 (2013) 95–103

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Characterization and seasonal changes in LHβ and FSHβ mRNA of *Rhinella arenarum* (Amphibia, Anura)

M. Clara Volonteri *, Eleonora Regueira, M. Florencia Scaia, Nora R. Ceballos

Laboratorio de Endocrinología Comparada, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 26 September 2012
Revised 13 March 2013
Accepted 17 March 2013
Available online 22 April 2013

Keywords:
Pituitary
Gonadotropins
Toad
Seasonal variations
Androgens

ABSTRACT

In anurans, two types of gonadotropins were described in several species of Ranidae and Pipidae families but only in one of the Bufonidae family. *Rhinella arenarum* is a bufonid that have the lowest concentration of plasma androgens during the breeding. The objective of this paper was to characterize the cDNA sequence of β subunit of LH and FSH from toad pituitary and study seasonal variation in gonadotropins mRNA using quantitative real-time RT-PCR. The LHβ cDNA is a 636 bp sequence containing an open reading frame (ORF), 45 bp of 5′-untranslated region (UTR) and 174 bp of 3′-UTR. The ORF encodes for a signal peptide of 26 amino acids and a mature protein of 113 amino acids with one N-glycosylation site at the 34th position. The FSHβ cDNA sequence is a 535 bp fragment containing an ORF, 8 bp of 5′-UTR and 152 bp of 3′-UTR. The ORF encodes for a signal peptide of 20 amino acids and a mature protein of 104 amino acids with two N-glycosylation sites at 25th and 42nd positions. Multiple alignments of aminoacid deduced sequences of LHβ and FSHβ (teleosts, amphibians, birds, mammals) showed that all the tetrapods studied conserve 12 cysteins and one (LH) or two (FSH) N-Glycosylation sites. LHβ is closer to teleosts than to mammals and birds while FSHβ is closer to mammals. The analysis of seasonal changes in LHβ and FSHβ mRNA indicates that transcript levels have seasonal variations and that the profile of androgens is opposite to that of the gonadotropins mRNA.

1. Introduction

Pituitary gonadotropins play critical roles in the control of gametogenic and endocrine functions of vertebrate gonads (Kawauchi and Sower, 2006; Norris, 2007). The majority of vertebrate species expresses two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both gonadotropins as well as thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG) belong to a family of glycoprotein hormones composed by two non-covalently linked N-glycosylated subunits, the α-subunit, common for all the members of the family, and the β-subunit, specific for each active hormone (Norris, 2007; Pierce and Parsons, 1981).

Several authors have studied the importance of the N-glycosylation of these hormones and how the presence of carbohydrates affects hormonal functions (Baenziger et al., 1992; Manna et al., 2002). Baenziger et al. (1992) proposed that the glycosylation of the β subunits of LH plays a central role in hormone bioactivity by regulating the rate of hormonal clearance from the circulation without affecting the interaction with its receptor. Manna et al. (2002) found that two-point mutations in human LHβ, that provide an extra N-glycosylation consensus site, increase the bioactivity without affecting the affinity for the LH receptor.

In anurans, many papers have reported that bullfrog pituitary has two chemically distinct gonadotropins similar to mammalian LH and FSH (Hanaoka et al., 1984; Hayashi et al., 1992a,b; Licht and Papkoff, 1974; Licht et al., 1977; Papkoff et al., 1976; Takada and Ishii, 1984; Takahashi and Hanaoka, 1981, 1985). In addition, two types of gonadotropins were described in *Rana pipiens* (Zhang et al., 2007), *Xenopus laevis* (Huang et al., 2001), *Bufo japonicus* (Itoh et al., 1990; Itoh and Ishii, 1990; Komoike and Ishii, 2003), *Rana dybowskii* and *Rana nigromaculata* (Kim et al., 1998), and *Rana esculenta* (Pinelli et al., 1996).

In mammals, LH regulates androgen biosynthesis through the interaction with LH-receptor located in Leydig cells (Hall, 1994).

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; LHβ, β subunits of LH; FSHβ, β subunits of FSH; RIA, radioimmunoassay; UTR, untranslated region; ORF, open reading frame.

* Corresponding author. Address: Laboratorio de Endocrinología Comparada, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Pabellón 2, Ciudad Universitaria, C1428EHA Buenos Aires, Argentina. Fax: +54 11 4576 3384.

E-mail addresses: claravolonteri@bg.fcen.uba.ar (M.C. Volonteri), eleonoraregueira@gmail.com (E. Regueira), mflorenciascaia@bg.fcen.uba.ar (M.F. Scaia), nceballo@bg.fcen.uba.ar (N.R. Ceballos).

0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.03.025

In amphibians, gonadotropins are also involved in the regulation of testicular steroidogenesis (Cobellis et al., 2008; Kobayashi et al., 1993; Licht et al., 1983; Pozzi et al., 2006; Pozzi and Ceballos, 2000). However, in several anuran species plasma androgens decline in spring, when reproduction takes place, reaching the lowest values in the summer (Guarino et al., 1993; Itoh et al., 1990; Rastogi et al., 1986), even though LH and FSH rise during the reproductive season (Kim et al., 1998; Licht et al., 1983; Polzonetti-Magni et al., 1998). The fact that plasma gonadotropins increase when androgens decline suggest that some aspects of the regulation of steroidogenesis and gonadotropin synthesis and secretion remain to be explored. Nevertheless, even if the importance of pituitary gonadotropins for steroidogenesis is accepted, little is known about the mechanisms whereby gonadotropins induce this process (Nagahama, 1986).

Rhinella arenarum is a South American toad with a breeding season restricted to springtime i.e., between September and December, and it is characterized for having the lowest concentration of plasma androgens during the breeding (Canosa and Ceballos, 2002a; Canosa et al., 1998; Denari and Ceballos, 2005). Besides, it was suggested that FSH is involved in the regulation of the change in testicular steroidogenesis during the breeding (Canosa and Ceballos, 2002b).

For a better understanding of the reproductive physiology of amphibians, it is important to take into account seasonal changes in the reproductive events, among them the annual variation in gonadotropins synthesis. Taking these considerations into account, the main objective of this paper is to characterize the cDNA sequence of LHβ and FSHβ subunits from *R. arenarum* pituitary as well as to study the seasonal variation in the expression of β subunits mRNA of both gonadotropins by using quantitative real-time RT-PCR method.

2. Materials and methods

2.1. Materials

All the primers used and the GeneRacer Kit were from Invitrogen (Carlsbad, CA). GenElute™ Mammalian Total RNA Miniprep Kit was acquired in Sigma–Aldrich (St. Louis, MA). Oligo(dTs) were obtained in Biodynamics (Buenos Aires, Argentina). RNase-free DNaseI, RT-buffer, dNTPs mix, AMV Reverse Transcriptase, $MgCl_2$ and Go*Taq* DNA Polymerase were purchased in Promega (Madison, WI). Accuprep purification kit was from Bioneer (Daejeon, Korea) and FastSart Universal SYBR Green Master (Rox) was from Roche (Mannheim, Germany). Tricaine methanesulfonate (MS222) was obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Animals

Reproductive male toads of *R. arenarum* were collected near Buenos Aires City during all year long. Animals were maintained with free access to water and fed with crickets and zophobas under natural conditions of light and temperature. For tissue preparation, animals were over-anaesthetized by immersion in 1% aqueous solution of MS222 (Gentz, 2007). To avoid the variability due to diurnal changes in hormone production, both pituitary and blood were always obtained at the same time of the day (between 10 and 12 am). 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 Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.

2.3. Tissue collection and total RNA isolation

For gonadotropin characterization and tissue expression studies, brain, spinal cord, heart, liver, kidney, adrenal gland, lung, testis, Bidder organ and pituitary from different animals were rapidly excised and stored at −70 °C until total RNA isolation. Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit, RNA samples were quantified with the Qubit Fluorometer (Invitrogen, Carlsbad, CA) and RNA integrity was checked by 1% agarose gel electrophoresis. All RNA samples were pretreated with RNase-free DNaseI to remove genomic DNA contamination prior to reverse transcription.

2.4. Reverse transcription (RT)

Transcription of RNA into cDNA was carried out by reverse transcriptase reaction as follows: 0.5 μg of RNA was incubated with 0.3 μl oligo(dT) primer (sequence: 5′-TTT TTT TTT TTT TTT-3′) in a 20 μl final volume, at 70 °C for 10 min. After incubation, samples were placed rapidly on ice. Reverse transcription was performed by adding to each sample a mixture of DEPC treated water, 4 μl AMV RT-buffer, 2 μl dNTPs mix (25 mM each), and 0.2 μl AMV Reverse Transcriptase. The incubation was carried out at 48 °C for 45 min and ended by heating at 95 °C for 5 min.

2.5. Oligonucleotides

Oligonucleotides used as PCR primers for the 3′ and 5′ rapid amplification of cDNA ends (RACE) and for Real time-PCR are listed in Table 1. Degenerate primers were designed using a Basic Local Alignment Tool (BLAST) based on the known nucleotide sequences of different species (see Table 2 for GenBank Accession numbers).

All specific primers used for real time PCR were synthesized based on LHβ, FSHβ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, expression control) sequences obtained from the degenerate PCR or 3′ and 5′ RACE.

Table 1
Sequence of oligonucleotide primers used.

Primer	Sequence
LH-F	5′-GAGAARGAVSRCTGYCCA-3′
LH-R	5′-TSARWGCCACRGGGTAGG-3′
FSH-F	5′-TGGTGYKCMGGATACTGC-3′
FSH-R	5′-TCCACARTGACARTCRAC-3′
GAPDH-F	5′-ACATGTTCMARTATGAYTC-3′
GAPDH-R	5′-AAKTTGTCRTKGATGACCT-3′
LH-3′ RACE	5′-CCTGGTTGCTTACTTGTCTGCCGTGC-3′
LH-3′ nested RACE	5′-CCATGTCATCTCGTCAATGCCACCA-3′
LH-5′ RACE	5′-TGGTGGCATTGACGAGATGACATGG-3′
LH-5′ nested RACE	5′-GCACGGCAGACAAGTAAGCAACCAGG-3′
FSH-3′ RACE	5′-GGCTGTCCTGAGAAAGTAAATCCTT-3′
FSH-3′ nested RACE	5′-TCCGGTGGCGGTTGACTGTCACTGT-3′
FSH-5′ RACE	5′-ACAGTGACAGTCAACCGCCACCGGA-3′
FSH-5′ nested RACE	5′-AAGGATTTACTTTCTCAGGACAGCC-3′
LH-F-SP	5′-TCCTTGGTCCTGGTTGCTTACTTG-3′
LH-R-SP	5′-GTGGTCCTTCTCGGCAGATATGG-3′
FSH-F-SP	5′-GAGATTGTGCCTGTATTATGTTC-3′
FSH-R-SP	5′-GATGGTGGAATTGCTCTTTAAC-3′
GAPDH-F-SP	5′-CCCATCACCGTCTCTCAGG-3′
GAPDH-R-SP	5′-CGCTTGGCACCTCCTTTC-3′
GeneRacer™ 5′ Primer	5′-CGACTGGAGCACGAGGACACTGA-3′
GeneRacer™ 5′ Nested Primer	5′-GGACACTGACATGGACTGAAGGAGTA-3′
GeneRacer™ 3′ Primer	5′-GCTGTCAACGATACGCTACGTAACG-3′
GeneRacer™ 3′ Nested Primer	5′-CGCTACGTAACGGCATGACAGTG-3′

IUB code: R = AG, Y = CT, K = GT, M = AC, S = GC, W = AT, B = CGT, D = AGT, H = ACT, V = ACG, N = AGCT. SP = specific primer.