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Homologous desensitization and visualization of the tilapia GnRH type 3 receptor

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Abstract

Two types of gonadotropin-releasing hormone (GnRH) receptors were found in the pituitary of tilapia (t), named GnRHR type 3 (tGnRHR3) and GnRHR type 1, according to phylogenetic analysis. tGnRHR3 is highly expressed in the posterior part of the pituitary which contains LH and FSH cells. We characterized tGnRHR3 in terms of both LH release rate and receptor internalization rate in response to continuous exposure to GnRH.

Constant exposure of tilapia pituitary fragments to salmon GnRH analog (sGnRHa) resulted in an increased secretion rate for 3 h, followed by a gradual decline, taking 17–19 h, to the basal secretion rate. A chimera between tGnRHR3 and green fluorescent protein (GFP) was created and used to observe the changes in receptor distribution and translocation, activated by agonist with time. The results suggested that the receptor is initially localized at the plasma membrane and upon activation by a homologous ligand (e.g. sGnRHa) undergoes relatively rapid endocytosis. In summary, the present work demonstrates that tGnRHR3 has already undergone endocytosis after 30 min, while desensitization of LH release occurs only after 17–19 h. It is concluded that for tGnRHR3, internalization of the receptor is not exclusively responsible for the desensitization of LH release.

Keywords: Pituitary; Tilapia; Internalization; Desensitization; Receptor; GnRH; GFP; Perifusion

1. Introduction

Gonadotropin-releasing hormone (GnRH) plays a critical role in the control of reproductive functions, in both mammals and teleosts, by stimulating the biosynthesis and secretion of the gonadotropins LH and FSH from the pituitary (Yaron and Levavi-Sivan, 2006). In fish, which do not exhibit a hypothalamo-hypophyseal portal blood system, GnRH is synthesized in the hypothalamus and the hypothalamic GnRH nerve fibers directly innervate the anterior pituitary, where the GnRH binds to a specific high-affinity receptor (Yaron et al., 2001, 2003). Most vertebrate species exhibit two or more forms of GnRH (Fernald and White, 1999); in perciform fish, such as tilapia, three forms of GnRH are evident: chicken GnRH-II (GnRH2), located in the neurons within the midbrain tegmentum; salmon GnRH (GnRH3) located in the terminal nerves, and sea bream GnRH (GnRH1), present in the preoptic area of the hypothalamus and in the pituitary (Gothilf et al., 1996; White et al., 1995). The latter is thought to be the main form inducing the release of gonadotropins from the pituitary (Carolsfeld et al., 2000; Powell et al., 1995). The presence of various forms of GnRH prompted an investigation, which led to the identification of more than one receptor (Troskie et al., 1998).

According to their sequences, the GnRH receptors can be grouped into distinct types: 1, 2, and 3 (Millar et al.,

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2004), which can be segregated into three branches. One branch contains all type-1 GnRH receptors (GnRHR1) from mammals and fish; a second branch clusters mainly amphibian and human type-2 GnRH receptors (GnRHR2); and a third branch includes type-3 GnRH receptors (GnRHR3) of modern teleost fish, mainly perciform species (Levavi-Sivan and Avitan, 2005). According to this classification, two types of GnRHRs were found in the pituitary of tilapia, named GnRHR1 and GnRHR3 according to phylogenetic analysis (nomenclature as in (Levavi-Sivan and Avitan, 2005) and (Millar et al., 2004)). In tilapiines such as astatotilapia (*Haplochromis burtoni*) or the Nile tilapia (*Oreochromis niloticus*), distributions of GnRHR1 and GnRHR3 within the same species are distinctly different. GnRHR3 is found mainly in brain areas related to reproductive function and is highly expressed in the posterior part of the pituitary which contains LH and FSH cells, suggesting that this receptor type may be important for regulating reproduction. Conversely, GnRHR1 is detected widely throughout the brain, from the olfactory bulb to the medulla, as well as in the dorsal-anterior and posterior parts of the pituitary, suggesting that this receptor type is in a position to allow GnRH modulation of sensory input and growth (Chen and Fernald, 2005; Parhar et al., 2002, 2005). Moreover, gene-expression levels of tGnRHR3 were increased both *in vivo* and *in vitro* in response to salmon GnRH analog (Levavi-Sivan et al., 2004), and after exposure to low doses of either estradiol or 17α, 20β, dihydroxy-4-pregnen-3-one (Levavi-Sivan et al., 2006). In mammals, the reproductive type of GnRHR is classified as type 1 (Millar et al., 2004). The present work will focus on the reproductive type of tilapia GnRHRs, namely tGnRHR3.

GnRHRs belong to the superfamily of seven-transmembrane G-protein-coupled receptors (GPCRs). In contrast to mammalian GnRHRs, the tilapia, like other non-mammalian GnRHRs, contain an intracellular carboxyl tail (C-tail) that is known to be important for ligand-dependent receptor desensitization and internalization (Heding et al., 1998; Lin et al., 1998; McArdle et al., 1999). Desensitization of the receptor starts with ligand binding right after the intracellular domain undergoes phosphorylation, which attenuates the interaction between the receptor and its G-protein. This desensitization may occur within seconds to minutes after agonist binding and is regarded as rapid homologous receptor desensitization. Thereafter, phosphorylation within the C-tail, by GRK (GPCR kinase), causes the recruitment of β-arrestin to the receptor phosphorylation sites and internalization via clathrin-coated vesicles, which are pinched off from the plasma membrane by dynamin (Ferguson et al., 1996; McArdle et al., 1999). This part of the desensitization occurs within minutes to hours of the stimulation. The lack of a C-tail in the mammalian receptor (Stojilkovic et al., 1994) prevents it from undergoing rapid homologous desensitization, as shown in αT3-1 cells (Davidson et al., 1994; Mcardle et al., 1995; Willars et al., 1998). The importance of the C-tail in the desensitization and internalization mechanism was hown by the addition of a C-tail from thyrotropin-releasing hormone receptor to the mammalian GnRHR, which led to rapid homologous receptor desensitization and internalization (Heding et al., 1998). Accordingly, truncation of the C-tail from the chicken GnRHR caused slow internalization (Pawson et al., 1998).

In mammals, physiological secretion of GnRH occurs in a pulsatile manner. However, sustained exposure to GnRH is known to desensitize gonadotropin secretion in response to GnRH stimulation (Smith and Vale, 1981). This homologous desensitization underlies the clinical application of GnRH analog for reproductive-system suppression (Barbieri, 1992).

Chicken GnRHRs, like all non-mammalian GnRHRs, possess a C-tail which facilitates rapid internalization of the receptor (Pawson et al., 1998). This rapid receptor internalization is in agreement with the observation that continuous exposure of chicken anterior pituitary cells to GnRH causes rapid desensitization in LH secretion (King et al., 1986). Xenopus GnRHRs also exhibit a C-tail, which promotes rapid internalization (Hislop et al., 2000). However, the secretion of LH from pituitary fragments of a ranid frog, in response to continuous exposure to GnRH, does not demonstrate desensitization in LH secretion (Porter and Licht, 1985).

Many studies have been conducted on continuous LH release, in several vertebrate species, and on internalization rate of the different types of GnRHR. Since in tilapia, GnRHR3 is known to be expressed on the gonadotrophs, and hence involved in GtH release, while in mammals and chicken the GnRHR type involved in GtH release is GnRHR type 1, we sought to describe the internalization rate of GnRHR3 and the desensitization rate of LH release from pituitary fragments in tilapia.

2. Materials and methods

2.1. Fish

Tilapia hybrids (*Oreochromis niloticus x O. aureus*) were collected from local kibbutz fish farms and housed at the university's fish facility, in 500-L tanks under a natural photoperiod and 26 ± 2 °C. The fish used in the experiments (weighing 50–150 g) were sexually mature. They were fed every morning *ad libitum* with commercial pellets and flakes containing 50% protein, 6% fat, 5.6% ash and 2.6% cellulose (Zemach Feed Mills, Zemach, Israel). All experimental procedures were in compliance with the Animal Care and Use Guidelines at the Hebrew University as approved by the local administrative panel of the Laboratory Animal Care Committee.

2.2. Perifusion of tilapia pituitary fragments

Perifusion was performed as previously described by Levavi-Sivan and Yaron (1989) and Levavi-Sivan et al. (1995). Briefly, pituitaries were excised from three fish and cut into fragments. The fragments were embedded in Biogel P-2 and perifused at 26 ± 1 °C with Eagle's basal medium for 14 h before beginning the experiment, in order to reach a steady low baseline. The average LH secretion rate, during the last 3 h of rinsing, was taken as the baseline. Salmon GnRH analog ([D-Ala^6,Pro^9-NEt]-

mammalian GnRH; sGnRHa; Bachem Inc., Torrance, CA) was introduced into the medium. The medium was sampled once an hour and tilapia LH (taGtH) was determined by specific radioimmunoassay as previously described (Levavi-Sivan and Yaron, 1989). LH release is expressed as means ± SEM ($n = 3$ channels/treatment) of the ratio between the secretion rate after manipulation and the basal one. The experiments were repeated three times and a representative experiment is shown.

2.3. Statistical analysis

Statistical analyses of differences in LH secretion rate between different sGnRHa doses were performed using ANOVA for repeated measurements, followed by Student Newman Keuls post test, using PRISM 4.02 software (GraphPad, San Diego, CA).

2.4. Construction of tilapia GnRHR type 3 fused to GFP

The entire open reading frame (ORF) of tGnRHR3 (GenBank Accession No. AY381299) was cloned into pEGFP-N1 (Clontech, Palo Alto, CA), giving rise to ptGnRHR3-GFP. In order to fuse the receptor to the reading frame of the green fluorescent protein (GFP), PCR was performed using the forward primer GCCACCATGAATGCCTCTCT GTGTGACCC, which contains the first eight amino acids of the ORF of tGnRHR3 together with a kozak sequence in order to enhance protein expression, and the reverse primer GAATTCCTAAGATGCTCTCAGC ACTGGA, in which the receptor's stop codon was eliminated and replaced with the amino acid glycine, and an EcoRI site was inserted (underlined). The receptor was subcloned into pGEM-T Easy (Promega, Madison, WI). The fragment was then digested with EcoRI and cloned into the same site of the expression vector pEGFP-N1 (Clontech). Clones were verified by sequence analysis (Sequencing Unit, The Weizmann Institute of Science, Israel).

2.5. Transient transfection of COS-7 cells

A COS-7 cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained at 37 °C in DMEM containing 4500 mg/ml glucose (Gibco, BRL, Paisley, UK) supplemented with 10% (w/v) fetal bovine serum (Gibco), 1% (w/v) glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Biological Industries, Beith Haemek, Israel), under 5% CO_2 until confluent. Twenty-four hours prior to transfection, COS-7 cells were seeded in 12-well culture plates (Nunc, Roskilde, Denmark) at a density of 4×10^4–6×10^4 cells/well. For confocal microscopy, the same amount of cells were seeded on glass coverslips (24 × 40 mm) that had been rinsed twice with 100% ethanol, and placed in 12-well culture plates.

Transfection was carried out using FuGENE 6 reagent (Roche Applied Science, Mannheim, Germany) as described by Levavi-Sivan et al. (2005). Twenty-four hours prior to stimulation, cells were starved of serum by replacing the standard medium with serum-free medium containing 0.5% (w/v) bovine serum albumin (BSA).

2.6. Detection of fusion protein by Western blot analysis

COS-7 cells were transiently transfected with ptGnRHR-3-GFP or with pEGFP-N1 (Clontech; 6 μg plasmid per six-well culture plate). Cells were harvested 48 h after transfection, with lysis buffer made up of 150 mM sucrose, 80 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1% (w/v) Triton X-100, 1 mM PMSF, and 40 μg/ml protease inhibitor cocktail. Samples were centrifuged for 15 min at 12,000*g*, and the supernatants were mixed with 5×-concentrated Laemmli sample buffer. Samples were boiled for 10 min prior to resolving on a 10% SDS–polyacrylamide running gel, with 5% stacking gel. Samples were electrotransferred to nitrocellulose membranes (Schleicher and Schuell, Dassell, Germany). Membranes were blocked for 1 h with 1% BSA (Sigma, Ness Ziona, Israel) in TBS-Tween (Tris–HCl 20 mM, pH 7.4; NaCl 137 mM; Tween 20 0.01%). Blots were incubated with primary antibody—anti-GFP mAb (Roche Applied Science, 1:1000) in 0.1% BSA in TBT-T overnight at 4 °C with gentle agitation, then incubated for 2 h with a secondary antibody—goat anti-mouse horseradish peroxidase conjugate (Jackson Immuno Research Laboratories, West Grove, PA; 1:40,000) in 0.1% BSA in TBS-T. After washing, the signal was enhanced with chemiluminescence reagent (ECL, Biological Industries).

2.7. Confocal microscopy

Twenty-four hours after starvation, cells were stimulated with sGnRHa for 0, 10, 30, 60 or 120 min. After stimulation, cells were washed twice with PBS and fixed with cold methanol for 5 min. Fixed cells can be exposed to more laser light than live ones, without visible damage (Cornea et al., 1999). Coverslips were removed from the 12-well culture plate and mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For longer preservation, coverslips were permanently sealed around the perimeter with nail polish and kept protected from light, at 4 °C. Observations were made using a Zeiss 510 LSM confocal microscope (Jena, Germany) with a 488-nm Argon excitation laser. Specimens were examined under a 63× water objective with additional zoom. Single or optical sectioning and projection of each cell was used. All images were taken with the same parameters of pinhole, gain and off-set. All experiments were repeated three times. Ten different fields were screened per coverslip, and three coverslips were analyzed from each experiment. A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Continuous exposure of tilapia pituitary fragments to GnRH

To estimate the desensitization of pituitary cells to GnRH stimulation in terms of LH release, we exposed perifused pituitary fragments to continuous GnRH stimulation for 24 h. Continuous exposure to 10 or 100 nM sGnRHa stimulated an initial 19- or 28.5-fold increase, respectively, in LH release within the first 3 h (Fig. 1). LH secretion rates decreased slowly thereafter, reaching baseline only after 17 or 19 h, for 10 or 100 nM sGnRHa, respectively. LH secretion rates measured after exposure to 10 and 100 nM sGnRHa were statistically different, relative to each other as well as to LH secretion rates in the control group. A further significant increase (4.35-fold over basal level; lasting 2 h), in response to a second short pulse of sGnRHa, confirmed that the gradual decrease was not due to depletion of LH stores within the pituitary. This latter increase, in response to a 5-min stimulation of 10 nM sGnRHa, was of the same magnitude (both in terms of secretion rate and duration) as that reported by us previously, for the same concentration of sGnRHa given as a first stimulation (Levavi-Sivan and Yaron 1992, 1993).

3.2. Validation of the tGnRHR-3-GFP

To visualize and follow the translocation of tGnRHR3 following stimulation with GnRH, we fused the receptor to GFP, giving rise to ptGnRHR3-GFP. To ensure the association between tGnRHR3 and GFP, cells were transiently transfected with ptGnRHR3-GFP, lysed and

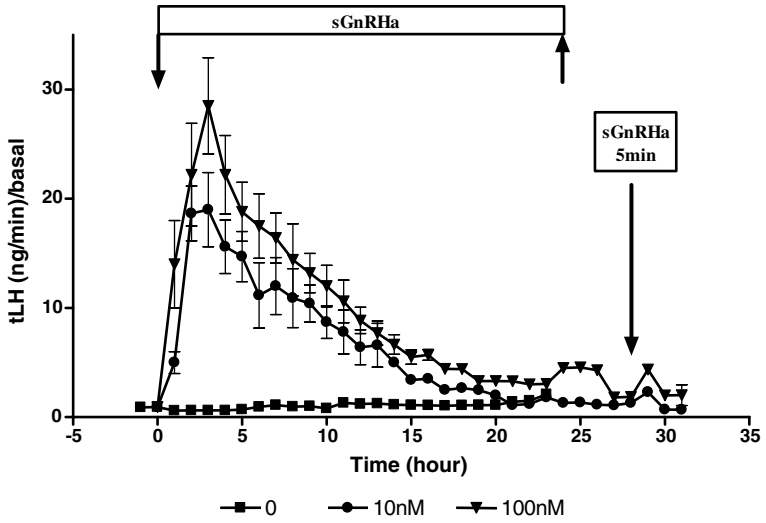

Fig. 1. Secretion of tilapia LH from perifused pituitary fragments exposed continuously for 24 h, or for 5 min, to sGnRHa (arrows) at 0 (squares) 10 nM (circles) or 100 nM (triangles). Data in this experiment are presented as means ± SEM ($n = 3$ channels/treatment) of the ratio between the secretion rate after stimulation and the basal secretion rate (the mean secretion rate during the last 3 h of rinsing in each respective channel).

separated by SDS–PAGE. Western-blot analysis performed with specific antiserum against the GFP, expressed by cells transfected only with pEGFP-N1 revealed that the size of the GFP was similar to the predicted size (27 kDa; Fig. 2, lane b), while the size of the fusion protein, tGnRHR3-GFP, was higher than predicted (110 vs. 80 kDa, respectively; Fig. 2, lane a). Since tGnRHR3 has four potential glycosylation sites at its N-terminus, (Levavi-Sivan and Avitan, 2005), the additional size probably reflects glycosylation of the fusion protein. The pharmacological activity of the receptor was determined in a signal-transduction assay and found to have the same ED_{50} as the native receptor (data not shown).

3.3. Visualization of receptor-internalization rate

To observe the changes in receptor distribution and translocation activated by agonist with time, several chamber slides with COS-7 cells expressing tGnRHR3-GFP were treated with 10 nM sGnRHa. Thereafter, the cells were fixed and visualized using a confocal microscope. At time zero, the receptors were detected on the cell surface (Fig. 3A); after 10 min stimulation with 10 nM sGnRHa, the receptor was visualized partially endocytosed from the internal surface of the cell membrane into the cytoplasm (Fig. 3B). After 30 and 60 min continuous stimulation, the receptor appeared to have detached from the membrane and was located in the cytoplasm, eventually accumulating in the perinuclear region (Fig. 3C and D). The degree of internalization did not change, even after 120 min of continuous exposure to the ligand (Fig. 3E).

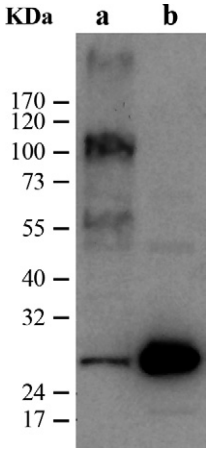

Fig. 2. Western-blot analysis with specific antisera against GFP. Extracts of cells transfected with ptGnRHR3-GFP (a) or pEGFP-N1 (b) were tested for the presence of a GFP product. Size markers are on the left.

4. Discussion

We characterized tGnRHR3 in terms of both LH release rate and receptor internalization rate in response to continuous exposure to GnRH.

Constant exposure of tilapia pituitary fragments to sGnRHa resulted in an increased secretion rate (up to 30-fold) for 3 h, followed by a gradual decline to the basal secretion rate, taking 17–19 h. A second exposure of the pituitary fragments to a 5-min pulse resulted in a peak of LH release which was similar in both its duration and secretion rate to that achieved previously when the same stimulation was given as a first stimulation (Levavi-Sivan and Yaron, 1992, 1993). These data strengthen the conclusion that the decrease in LH over time is not due to depletion of LH stores within the pituitary. The kinetics of the desensitization may vary in other vertebrates: continuous exposure of chicken pituitary to GnRH stimulates an initial 10-fold increase in LH release within minutes followed by a very rapid decrease to basal levels (within 2 h) (King et al., 1986). Continuous exposure of goldfish pituitary

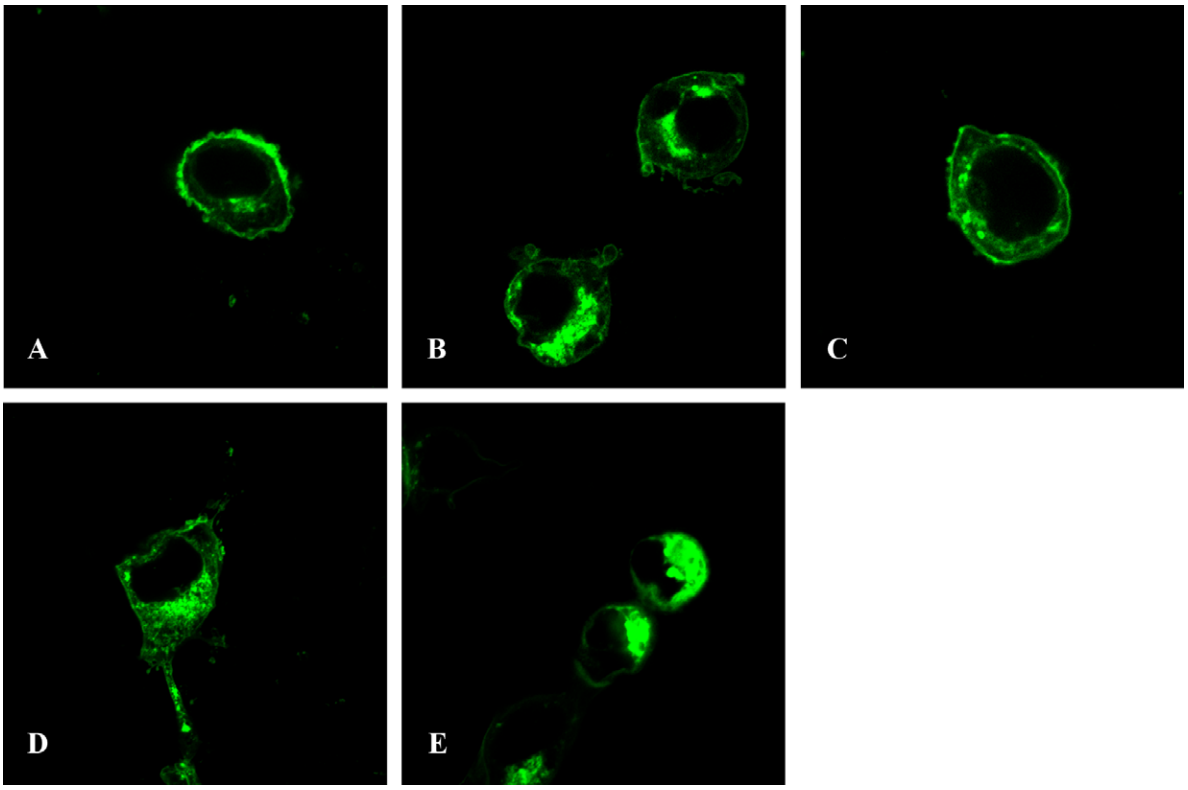

Fig. 3. Internalization of ptGnRHR3-GFP visualized by confocal microscopy. Cells were grown on coverslips, transiently transfected with ptGnRHR3-GFP, and stimulated with 10 nM sGnRHa for 10 min (B), 30 min (C), 60 min (D) or 120 min (E). Unstimulated cells serve as control (A). Cells were fixed on glass slides and visualized by confocal microscope using a 488-nm argon excitation laser, as described in Section 2.

fragments to sGnRHa resulted in an initial rapid increase (within 2–4 min; 10- to 15-fold) followed by a decline, reaching a steady state within 4–6 min (4-fold) (Habibi, 1991), while continuous exposure of catfish pituitary fragments resulted in slower (20–30 min; 2.5–fold) and prolonged (up to 3 h) increase in LH release (de Leeuw et al., 1986). In rats, sustained stimulation of GnRH causes a 20-fold elevation of LH release within 2 h followed by a slow decrease (return to basal level after 12 h) (Smith and Vale, 1981). However, continuous GnRH exposure of pituitary fragments of ranid frogs results in an increase in LH release in as little as 10 min, reaching a maximum within 8–14 h, and even after 20 h of stimulation, the level of LH release remains as high as in the first few hours of stimulation (Porter and Licht, 1985). These results show that except for ranid frogs, continuous GnRH exposure results in desensitization of pituitary LH release. It should be noted, however, that different species express different types of GnRHRs on their gonadotrophs. While gonadotropins are released through activation of GnRHR type 1 in mammals, chickens, catfish and goldfish, the gonadotrophs in tilapia possess GnRHR type 3 (Levavi-Sivan and Avitan, 2005).

Confocal microscopy was used to image cells expressing a fluorescent protein connected to tGnRHR3 upon stimulation by a specific ligand. The results suggested that the receptor is initially localized at the plasma membrane and upon activation by a homologous ligand (e.g. sGnRHa) undergoes relatively rapid endocytosis. In the present study, some aggregates of fluorescent signal were also seen in the cytoplasmic compartment, which may be the receptors at the sites of synthesis and trafficking. Previous studies using labeled GnRH agonist have shown that following internalization, the mammalian GnRHR becomes associated with either lysosomes, leading to degradation, or with the Golgi complex and hormone granules, leading to recycling (Childs, 1990; Schvartz and Hazum, 1987). The technique used in this study does not have sufficient resolution to localize the fluorescent signal within the cytoplamic compartment, and allow examination of the potential sites for receptor protein synthesis and trafficking. However, in a similar study in mammals, apparent internalization was observed only after 180 min (Cornea et al., 1999).

Unlike mammalian GnRHRs, tGnRHR3 has a C-tail, which is reported to be essential for rapid internalization and desensitization of GPCRs (Sealfon et al., 1997). Hence, it was not surprising to find that tGnRHR3 also exhibits rapid internalization of the receptor, as visualized by GFP and confocal microscopy. This finding corroborates earlier findings in which the internalization rate of a chimeric receptor consisting of rat GnRHR with the catfish C-tail was higher than that of the native rat GnRHR (Cornea et al., 1999; Vrecl et al., 2000).

Pawson et al. (1998) postulated that the lack of a C-tail may have been selected for to prevent rapid desensitization and internalization in the mammalian GnRHR, in order to allow the protracted LH surge (over several hours) required for oocyte maturation and ovulation. This is in contrast to the situation in chickens, which have only a one-day cycle including a substantially shorter LH surge of no more than 120 min (Furr et al., 1973). The situation in tilapia does not conform to this postulation inasmuch as the tilapia spawning cycle is relatively long (10–18 days) and the LH surge, which is associated with final oocyte maturation and ovulation, lasts at least 1 day (Aizen et al., 2007). We concluded, therefore, that the existence of a C-tail, which enables rapid receptor internalization, is not correlated with the duration of the pre-ovulatory LH peak.

In summary, the present work demonstrates that tGnRHR3 undergoes endocytosis within 30 min, while desensitization of the LH response occurs only after 17–19 h. It is concluded that for tGnRHR3, internalization of the receptor is not exclusively responsible for the desensitization of LH release.

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