Review Article

Activating and inactivating mutations of the human, rat, equine and eel luteinizing hormone/chorionic gonadotropin receptors (LH/CGRs)

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ABSTRACT Mutations in the luteinizing hormone/chorionic gonadotropin receptors (LH/CGRs), representatives of the G protein-coupled receptor family, have been rapidly identified over the last 20 years. This review aims to compare and analyze the data reported the activating and inactivating mutations of the LH/CGRs between human, rat, equine and fish, specifically (Japanese eel *Anguilla japonica*). Insights obtained through detailed study of these naturally-occurring mutations provide a further update of structure-function relationship of these receptors. Specifically, we present a variety of data on eel LH/CGR. These results provide important information about LH/CGR function in fish and the regulation of mutations of the highly conserved amino acids in glycoprotein hormone receptors.

Keywords: activating LH/CG receptor, fish (eel), inactivating LH/CGR, mammalian

INTRODUCTION

Gonadotropin receptors, such as luteinizing hormone/chorionic gonadotropin receptor (LH/CGR), belong to a large superfamily of G protein-coupled receptors (GPCRs) characterized functionally by their interaction with guanine nucleotide-binding proteins and structurally by their seven transmembrane spanning domains, extracellular amino-terminus, and intracellular carboxy-terminus (Min et al., 1998; Byambaragchaa et al., 2021a).

GPCRs exert their effects through G proteins, found at the cytoplasmic face of a cell's plasma membrane. G proteins are composed of three subunits: alpha (α), beta (β), and gamma (γ). The $G\alpha$ subunit contains a highly conserved GTPase domain. The G protein β - and γ -subunits form a functional unit that can only be dissociated under denaturing conditions. Different combinations of these three subunits are important in governing the diverse signaling pathways regulated by GPCRs (Oldham and Hamm, 2008). These $G\alpha$ subunits are divided into four classes:

$G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$ (Syrovatkina et al., 2016). $G_{\alpha s}$ and $G_{\alpha i}$ transmit signals by stimulating or inhibiting adenylate cyclase, respectively, thereby controlling cyclic AMP levels.

The receptors of glycoprotein hormones use two specific pathways. The cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway which produces cAMP via adenylate cyclase. The protein kinase C pathway works in concert with the Ca^{2+} -dependent activation of phospholipase C (Byambaragchaa et al., 2021a). These pathways then initiate intracellular phosphorylation cascades to control a diverse range of processes in growth and metabolism (Ascoli, 2007; Sente et al., 2018).

Similar to other GPCRs, the agonist-induced activation and subsequent phosphorylation of LH/CGRs are important steps for agonist-induced internalization (Levoye et al., 2015; Byambaragchaa et al., 2021a). These receptors are distributed throughout the plasma membrane, but upon agonist activation, they cluster in clathrin-coated pits. The clustered agonist-receptor complex is internalized and moves to the endosomal compartment without ligand dissociation (Lazari et al., 1998; Slosky et al., 2020; Jones et al., 2021). Most of the internalized receptor-agonist complexes are transported to the lysosome where the agonist and receptor are degraded (Kishi et al., 2001). In some cases, the complex accumulates in endosomes and the receptor is later recycled back to the cell surface where it is again available to bind to agonist (Krishnamurthy et al., 2003; Foster et al., 2019; Zhou et al., 2021). Whether the number of available cell surface receptors are reduced or maintained is determined by these internalized gonadotropin receptor recycling or degradation processes (Galet et al., 2003, 2004; Hirakawa and Ascoli, 2003; Foster et al., 2017; Byambaragchaa et al., 2020, 2021a). Receptor recycling promotes the maintenance of cell surface receptors and preserves responsiveness to agonists such as hormones (Bhaskaran and Ascoli, 2005; Byambaragchaa et al., 2021a,b).

The first naturally-occurring GPCR mutation was reported due to study of the human disease it causes (Dryja et al., 1990). Excellent reviews on GPCRs in general have been published (Themmen and Huhtaniemi, 2000; Schöneberg et al., 2004; Spiegel and Weinstein, 2004; Themmen, 2005; Tao, 2006, 2008; Althumairy et al., 2020) together with many reviews specifically concerning mutations in individual GPCRs. In this review, we focus on the

biological activity of cAMP signal transduction following agonist treatment, cell-surface loss of receptors, and summarizing the impact of receptor mutations in the fish (Japanese eel *Anguilla japonica*) LH/CGR on these processes. These comparative data reveal how the activating/inactivating mutants both of mammalian and fish affect signal transduction in the LH/CGR-LH or eCG complex in the highly conserved region of LH/CGR. New studies are just beginning to reveal an important signal transduction for GPCR pathway.

Activating mutations

LH/CG receptor mutations can activate precocious puberty in boys, whereas, inactivating mutations can cause anovulation in women and block sexual maturation in men (Themmen and Huhtaniemi, 2000). Recently, we reported the structure-function of equine LH/CGR (eLH/CGR) in cells expressing the eLH/CGR activating gene (Byambaragchaa et al., 2021a), as shown in Fig. 1. Four constitutively activating mutants (M398T, L457R, D564G, and D578Y), spontaneous mutations in the human LH/CGR, were analyzed for cAMP response and cell-surface receptor loss. The eLH/CGR-L457R, -D564G, and -D578Y mutants exhibited 16.9-, 16.4-, and 11.2-fold increases in basal cAMP response, respectively. The eLH/CGR-M398T mutant showed a slight increase (1.4-fold) without agonist treatment. The activating mutants showed rapid rates of cell surface loss of the receptor.

In humans, the LH/CGR (hLH/CGR), hLH/CGR-D578G mutant (equivalent to D578G or D578Y in eLH/CGR), which is inherited in an autosomal dominant male-limited pattern, produced a 4.5-fold increase in basal cAMP responsiveness in COS-7 cells (Shenker et al., 1993; Kosugi et al., 1996). This demonstrated that it was constitutively active and represented approximately 42% of the maximal stimulation of the wild-type receptor. The other mutant, D564G in the third intracellular loop, also had elevated basal cAMP production (Laue et al., 1995; Kosugi et al., 1998). The M398T mutant, located in the second transmembrane region, exhibited the same constitutively high basal cAMP levels (Yano et al., 1996), showing that the mutant's cAMP response in the absence of hormone was elevated up to 25-fold compared to the response of the wild-type receptor (Kraaij et al., 1995). Specifically, the basal cAMP response of the hLH/CGR-L457R mutant was dramatically increased, whereas the maximal cAMP

Fig. 1. Schematic representation of the structures of equine and eel LH/CGR (eLH/CGR and eelLH/CGR, respectively). The locations of the constitutive activating mutations (M398T, L457R, D564G, and D578Y) and the inactivating mutations (D405N, R464H, and Y546F) are indicated (left). In the eelLH/CGR, three constitutively activating mutations (M410T, L469R, and D590Y) and two inactivating mutations (D417N and Y558F) are indicated. Amino acid sequences at the mutated sites in the transmembrane domain and intracellular loop are shown in the eLH/CGR. In eelLH/CGR, the transmembrane domain II, III, V, and VI are shown. EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain; LH/CGR, luteinizing hormone/chorionic gonadotropin receptor.

response corresponded to only 40–50% of the maximal wild-type receptor response (Zhang et al., 2005, 2007; Galet and Ascoli, 2006; Latronico and Segaloff, 2007). Mutations in the transmembrane helix 6 and the third intracellular loop at position 578 are most commonly mutated to glycine (D578G) (Shenker et al., 1993; Laue et al., 1995).

In rats, the LH/CGR-D556G mutant (rLH/CGR-D556G: equivalent to D578Y or D578G in eLH/CGR) is constitutively activating like the D578G mutant eLH/CGR (Bradbury et al., 1997). Therefore, the mutant protein is not processed in the trans-Golgi and remains in its 73 kDa form, remaining predominantly in the endoplasmic reticulum. rLH/CGR-L435R (equivalent to L457R in eLH/CGR) exhibited a 25-fold increase in basal cAMP and enhanced the internalization of agonist-occupied receptors 17-fold (Min et al., 1998). The phenotype of female knock-in mice in the LH/CGR-D582G has been reported to be distinctly different from women with activating mutations, indicating that the female mice are normal (Narayan, 2015). Knock-in mice undergo precocious puberty and are infertile with significant ovarian diseases, such as hemorrhagic cysts, cell hyperplasia, and granulosa cell tumors (Hai et al., 2015).

In eel, we first reported that the activating mutants (eelLH/CGR-M410T, -L469R, and -D590Y) exhibited a 4.0-, 19.1-, and 7.8-fold increase in basal cAMP response without agonist treatment (Byambaragchaa et al., 2021c), indicating that these mutants were found to rapidly cause cell-surface loss of receptors. The loss rates of cell surface

agonist-receptor complexes were observed to be very fast (2.6–6.2 min) in both the wild-type eel LHR and the activating mutants (in press). We also suggested that the activating mutants in the eel follicle-stimulating hormone receptor (eelFSHR) were consistent, demonstrating that, compared to the wild-type, the basal cAMP response increased considerably and the loss of cell-surface receptor decreased quickly (Byambaragchaa et al., 2020).

Inactivating mutations

In the testis, LH/CGR is expressed in the Leydig cells, whereas the ovary expresses it in the theca/granulosa cells (Tao, 2006). Thus, loss of function mutations in LH/CGR lead to Leydig cell hypoplasia in males. To date, many studies have reported the effects of eLH/CGR-D405N, eLH/CGR-R464H, eLH/CGR-Y546F (Byambaragchaa et al., 2021a). In the hLH/CGR, many mutations have been reported that hLH/CGR-V144F (Richter-Unruh et al., 2005), hLH/CGR-F194V (Gromoll et al., 2002), hLH/CGR-N291S (Laue et al., 1995), hLH/CGR-W491X (Richter-Unruh et al., 2002) and hLH/CGR-L502P in TM4 (Leung et al., 2004), and hLH/CGR-A593P and hLH/CGR-S616Y (Tao et al., 2004) have impaired trafficking to the plasma membrane. In rats, the inactivating mutants rLH/CGR-D383N (equivalent to D405N in eLH/CGR), rLH/CGR-R442H (R464H in eLH/CGR), and rLH/CGR-Y524F (Y546F in eLH/CGR) completely impaired signal transduction, cell surface loss of receptors, and internalization (Ji and Ji, 1991; Dhanwada et al., 1996; Min et al., 1998).

We also reported that the inactivating mutants, eelLH/

Fig. 2. G-protein signaling and β -arrestin-regulated internalization of GPCR. Ligands bind to receptor (a) and the phosphorylated receptors at the tail region combine with β -arrestin in place of G proteins (b). This is referred as β -arrestin recruitment (c). Next, β -arrestin recruit AP2 (adaptor complex) (d). After clathrin-coated vesicle formed (e), receptors with β -arrestin were internalized into the endosome (f). And then receptors are sorted to recycle back to the plasma membrane (g) and receptor in part degraded in the lysosome (h).

CGR-D417N (equivalent to D405N in eLH/CGR) and eelLH/CGR-Y558F (equivalent to Y546F in eLH/CGR), were impaired in cells expressing both mutant receptors compared to those expressing the wild-type receptor (Byamabaragchaa et al., 2021c). However, these mutants did not affect the basal cAMP response, but showed a slightly increased response under high concentrations of agonist. The loss of cell surface receptors in the cells expressing inactivating mutants D417N and Y558F was not observed, despite the treatment with a high concentration of agonist (1000–1500 ng/mL).

SUMMARY AND PERSPECTIVE

In conclusion, signal transduction of LH/CGRs occurs by PKA-mediated activation, demonstrating that activation mutations are stimulated by constitutively active LH/CGR mutants. This indicates that inactivating mutations in both mammalian and eel LH/CGRs are completely impaired in the PKA-mediated signaling pathway. However, the key pathway involved in PKA signaling should be examined in detail. The ERK1/2 cascade is a prominent mitogenic pathway activated by agonist-engaged wild-type LH/CGR. Thus, we propose that the LH/CGR-activated ERK1/2 cascade is necessary for the functional analysis of highly constitutively activating LH/CGRs. Primary cells expressing these mutants and animal models with knock-in of these genes will provide useful information about these important signaling pathways (β -arrestin signaling,

internalization, recycling, and downregulation, as shown in Fig. 2) by using new techniques based on the principle of time-resolved fluorescence resonance energy transfer or bioluminescence resonance energy transfer.

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