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The carboxy-terminal tail or the intracellular loop 3 is required for β-arrestin-dependent internalization of a mammalian type II GnRH receptor

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ABSTRACT

The type II GnRH receptor (GnRH-R2) in contrast to mammalian type I GnRH receptor (GnRH-R1) has a cytosolic carboxy-terminal tail. We investigated the role of β-arrestin 1 in GnRH-R2-mediated signalling and mapped the regions in GnRH-R2 required for recruitment of β-arrestin, employing internalization assays. We show that GnRH-R2 activation of ERK is dependent on β-arrestin and protein kinase C. Appending the tail of GnRH-R2 to GnRH-R1 enabled GRK- and β-arrestin-dependent internalization of the chimaeric receptor. Surprisingly, carboxy-terminally truncated GnRH-R2 retained β-arrestin and GRK-dependent internalization, suggesting that β-arrestin interacts with additional elements of GnRH-R2. Mutating serine and threonine or basic residues of intracellular loop 3 did not abolish β-arrestin 1-dependent internalization but a receptor lacking these basic residues and the carboxy-terminus showed no β-arrestin 1-dependent internalization. Our results suggest that basic residues at the amino-terminal end of intracellular loop 3 or the carboxy-terminal tail are required for β-arrestin dependent internalization.

1. Introduction

Gonadotropin-releasing hormone (GnRH) I is a hypothalamic peptide that regulates pituitary synthesis and secretion of the gonadotropins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), which regulate gonadal and reproductive function. Most eutherian mammals, including humans and marmoset monkeys, have a second form of GnRH, designated GnRH II. In contrast to GnRH I, which varies between mammals and non-mammalian vertebrates, the structure of GnRH II is completely conserved among jawed vertebrates (Millar et al., 2004, 2008). GnRH II has been reported to regulate reproductive behaviour and to modulate reproductive function at the pituitary and reproductive tissues and in addition, to have non-reproductive functions (Kauffman and Rissman, 2004; Millar et al., 2004, 2008; Sasaki and Norwitz, 2011; Urbanski, 2012). The mammalian pituitary type I GnRH receptor (GnRH-R1), which has high affinity for mammalian GnRH I and lower affinity for all other naturally-occurring forms of GnRH, is unique among G protein-coupled receptors (GPCRs) in lacking a cytoplasmic carboxy-terminal domain (Flanagan et al., 2007; Millar et al., 2004; Sefideh et al., 2014). Non-mammalian GnRH-R1 receptors are structurally homologous to mammalian GnRH-R1, but have carboxy-terminal domains like other GPCRs. Similarly, mammalian and non-mammalian type II GnRH receptors (GnRH-R2), which are selective for GnRH II, have a cytoplasmic carboxy-terminal domain (Flanagan et al., 2007; Millar et al., 2001, 2004; Neill et al., 2001; Sefideh et al., 2014).

All GnRH receptors activate the $G_{q/11}$ family of G proteins, which activate phospholipase C-catalyzed production of second messengers that activate protein kinase C (PKC). Mammalian GnRH-R1 activation of the extracellular signal-regulated kinases (ERK) 1 and 2 is dependent on activation of $G_{q/11}$ (Naor and Huhtaniemi, 2013) and it is well established that β-arrestins are not involved (Benard et al., 2001; Bonfil et al., 2004; Caunt et al., 2006a, 2006b; Dobkin-Bekman et al., 2006, 2009). On the other hand, activation of ERK 1 and 2 by the non-mammalian *Xenopus* GnRH-R1 is dependent on receptor interaction with β-arrestin in addition to activation of PKC. A chimaeric receptor consisting of the full length

Abbreviations: DMEM, Dulbecco's modified Eagle medium; EC_{50}, concentration that stimulates half-maximal IP production; ERK, extracellular signal-regulated kinases; FSH, follicle-stimulating hormone; GnRH-R1, Type I GnRH receptor; GnRH-R2, Type II GnRH receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinases; IC2, intracellular loop 2; IC3, intracellular loop 3; IC_{50}, half-maximal inhibitory concentration; IP, inositol phosphates; LH, luteinising hormone; PKC, protein kinase C; SDS, sodium dodecyl sulphate; TM, transmembrane helix.

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human GnRH-R1 ligated to the carboxy-terminal domain of the *Xenopus* GnRH-R1 also stimulated β-arrestin-dependent activation of ERK, showing that the appended carboxy-terminal tail confers β-arrestin activation on the chimaeric receptor and suggesting that it interacts with β-arrestin (Caunt et al., 2006a, 2006b). Agonist activation of GnRH-R2 stimulates phosphorylation of ERK (Millar et al., 2001), but the role of arrestin interaction has not been reported. β-Arrestin interaction with GnRH receptors has been more comprehensively studied via its role in receptor internalization. The mammalian GnRH-R1 does not exhibit rapid internalization. In contrast, various non-mammalian GnRH-R1 receptors showed rapid internalization that was largely thought to arise from β-arrestin interaction with their carboxy-terminal tails (Caunt et al., 2006a; Heding et al., 2000; Hislop et al., 2000; McArdle et al., 1999; Millar et al., 2008; Pawson et al., 1998; Vrecl et al., 1998; Willars et al., 1999).

There are four arrestin isoforms, the visual arrestins, arrestin 1 and 4, which are found only in the eye, and β-arrestin 1 and β-arrestin 2, also called arrestin 2 and 3, respectively, which are expressed ubiquitously. In the classical model of arrestin function, agonist activation of GPCRs results in activation and dissociation of heterotrimeric G proteins, which allows GPCR kinases (GRK) to phosphorylate serine and threonine residues on the cytosolic surface of the activated receptor. Arrestin binding to the phosphorylated GPCR blocks further interaction with the G protein, thus desensitizing cellular signalling to further agonist stimulation and GPCR-bound β-arrestin interacts with clathrin and the adaptor protein, AP2, to induce receptor clustering and internalization via clathrin coated pits (Gurevich and Gurevich, 2006). The GPCR-activated β-arrestin also provides a scaffold for G protein-independent signalling pathways, including activation of ERK (Gurevich and Gurevich, 2006). Arrestin binding to GPCRs involves at least two binding sites that recognize, respectively, the activated GPCR conformation and the negative charges of the phosphate groups on the GPCR cytosolic surface (Gurevich and Gurevich, 2006; Kim et al., 2013; Shukla et al., 2013). In most GPCRs, phosphate groups attached to serine or threonine residues in the carboxy-terminal tail or intracellular loop 3 (IC3) are recognized by conserved basic residues in the arrestins (Gurevich and Gurevich, 2006). This was confirmed by the recent crystal structure of activated β-arrestin in complex with a phosphorylated peptide corresponding to the carboxy-terminal tail of the V2 vasopressin receptor (Shukla et al., 2013). However, high affinity arrestin binding depends on additional contacts with non-phosphorylated GPCR elements that have largely not been characterized. Since both G proteins and arrestins interact preferentially with agonist-activated GPCRs and arrestin binding occludes G protein binding, it is thought that G proteins and arrestins both interact with a GPCR element that is accessible on the cytosolic surface only of the activated GPCR conformation (DeGraff et al., 2002; Gurevich and Gurevich, 2006). Crystal structures of activated GPCRs in complex with a heterotrimeric G protein or a G protein fragment show that G proteins bind to the inside surfaces of transmembrane helices (TM) 5 and 6, which are connected to each other by IC3 of the GPCR. Consistent with this, β-arrestin 2 binds to basic residues arginine and lysine of a "BxxBB" motif at the amino-terminal end of IC3 of the α_{2b} and α_{2c} adrenergic receptors, close to the cytosolic end of TM5 (DeGraff et al., 2002) and more recent evidence shows that visual arrestin interacts with the inner surfaces of TM5 and TM6 of activated rhodopsin (Sinha et al., 2014).

We have previously found that a mammalian (i.e. marmoset) GnRH-R2 is rapidly internalized in transfected COS-1 cells. The GnRH-R2 internalization was dependent on GRK2 but independent of β-arrestin. However the internalization was enhanced by co-expression of exogenous β-arrestin. Truncation of the carboxy-terminal tail or alanine substitution of two carboxy-terminal domain serine residues decreased GnRH-R2 internalization, whereas substitution of the serine residues with negatively-charged glutamate residues, which mimic phosphate, enhanced GnRH-R2 internalization. This showed that carboxy-terminal tail phosphorylation is necessary for rapid internalization of GnRH-R2. However, co-expression of β-arrestin 1 enhanced internalization of GnRH-R2 mutants lacking the phosphorylated serine residues or the carboxy-terminal domain, showing that these residues are not required for β-arrestin 1-dependent internalization of GnRH-R2 (Ronacher et al., 2004). This suggests that other domains of the GnRH-R2 also mediate β-arrestin 1-dependent internalization of GnRH-R2. GnRH-R2 has a BxBB motif at the amino-terminal end of IC3, which resembles the "BxxBB" of the α_{2b} and α_{2c} adrenergic receptors (DeGraff et al., 2002), and this motif is absent in GnRH-R1, suggesting that it may have a role in β-arrestin 1-dependent internalization of GnRH-R2.

In this study we have examined the role of β-arrestin in GnRH-R2-mediated activation of ERK and have employed receptor internalization assays to investigate the roles of the cytoplasmic carboxy-terminal tail and IC3 of GnRH-R2 in targeting the receptor to β-arrestin 1 dependent internalization.

2. Materials and methods

2.1. Generation of mutant and chimaeric GnRH receptor expression constructs

The human GnRH-R1 and the marmoset GnRH-R2 receptors were cloned into the expression vector, pcDNA3.1(+), between the *Eco*RI and *Xba*I restriction sites. Point mutations were introduced into the marmoset GnRH-R2 (Fig. 1A) employing bridge PCR-based site-directed mutagenesis, using Deep Vent® high fidelity DNA polymerase (New England Biolabs, UK) and mutagenic primers targeting the indicated amino acid positions (Figs. 1A, 2) and T7 (sense) and BGH (antisense) vector primers. Mutagenic primers contained the desired mutation and a silent restriction site for screening. PCR conditions were an initial 5 min at 94 °C and 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C. The final extension was performed at 72 °C for 10 min. For the bridge PCR, there was an initial

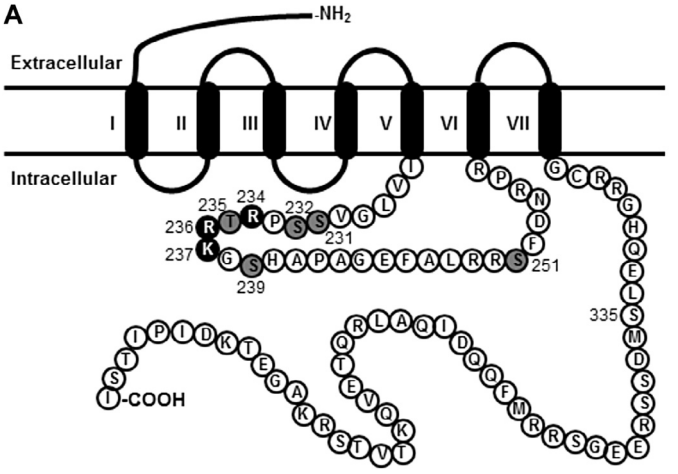

Fig. 1. Illustration of the wild type marmoset GnRH-R2 showing amino acid residues comprising IC3 and the cytosolic C-terminus. (A) Amino acids that were mutated in this study are numbered. Potential phosphorylation sites in IC3 are indicated in grey circles and basic residues at the amino-terminal end of IC3 in black. (B) Alignment of IC3 sequences of marmoset GnRH-R2 and human GnRH-R1. Basic residues that are not present in the GnRH-R1 and were mutated in this study are in bold.