**ARF6 activated by the LHCG receptor through the cytohesin family of guanine nucleotide exchange factors mediates the receptor internalisation and signalling**

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***Background.*** The LHCGR (Luteinizing Hormone Chorionic Gonadotropin Receptor) is a Gs-coupled GPCR that is essential for the maturation and function of the ovary and testis. LHCGR is internalised following its activation, which regulates the biological responsiveness of the receptor. Previous studies indicated that ARF6 and its GEF cytohesin 2 regulate LHCGR internalisation.[1] However, the mechanisms by which ARF6 and cytohesin 2 regulate LHCGR internalisation remain incompletely understood. Here we investigated the molecular details by which ARF6 regulates agonist-induced HLHCGR internalisation.

***Methods.*** We investigated the role of the ARF6 signalling pathway in the internalisation of heterologously expressed human LHCGR in HEK293 (HEK-HLHCGR) cells using ELISA, immunofluorescence and a combination of pharmacological inhibitors, siRNA and the expression of mutant proteins.[2] The functional activity of the agonist-occupied receptors was investigated by measuring cAMP accumulation and quantifying of the activate form of ARF6, ARF6-GTP, using a novel pull down assay.[3]

***Results*.** Depletion of ARF6 but not ARF1 significantly inhibited HCG-induced HLHCGR internalisation (~10% cell surface receptor loss in ARF6 siRNA transfected cells compared to ~30% in control or ARF1 siRNA transfected cells). HCG-stimulated cAMP accumulation was greater (8.3 $\pm$ 0.5 fold over basal) in ARF6 siRNA-transfected HEK-HLHCGR cells but not ARF1 siRNA (3.5 $\pm$ 0.2 fold over basal) or control siRNA (3.4 $\pm$ 0.3 fold over basal) transfected cells. These results confirm the role of ARF6 in agonist-induced HLHCGR internalisation and signalling. When HCG was added to the HEK-HCGLHR cells for 30 min, ARF6 was activated with increasing concentration of HCG and reached saturation at 100 IU/ml (3.1± 0.9 fold over basal). Furthermore, ARF6 activation in HCG-stimulated HEK-HLHCGR cells was specifically inhibited by the Myr-ARF6 peptide but not the Myr-ARF1 or control peptide. These data suggest a critical role for ARF6 activation in the internalisation of HLHCGR. Our further studies found that heterotrimeric G-protein, PI 3-kinase (PI3K), cytohesin ARF GEF and ARF GAP function upstream of ARF6 whereas dynamin and clathrin act down stream of ARF6 in the regulation of HCG-induced HLHCGR internalisation and signalling. These results suggest that the agonist binding to LHCGR results in Gαs activation, which then dissociates from Gβγ and activates adenylyl cyclase. Gβγ activates PI3K to produce the second messenger PIP3, which recruits cytohesin ARF GEFs to the membrane for ARF6 activation. We suggest that ARF6-GTP then activates PIP5K to produce PIP2, which recruits AP2 required for clathrin-coated pit assembly. ARF6-GTP also recruits NM23-H1 to clathrin-coated pits to provide GTP to dynamin dependent fission of clathrin-coated vesicles.

***Conclusions.*** We have identified the molecular mechanisms underlying the ARF6-dependent regulation of HLHCGR.

[1]Hunzicker-Dunn, et al., (2002). *FEBS letts.* **521:**3; [2]Lawrence et al., (2005). *Mol Pharmacol*. **67:**1822; [3]Venkateswarlu, K. (2005). *Methods in Enzymol.* **405:**252.

The work in VK's laboratory was funded by the BBSRC, MRC and the Royal Society.