Molecular coupling and phosphorylation profile of the orphan receptor GPR35

G protein-coupled receptor 35 (GPR35) is an orphan GPCR that has significant therapeutic potential due to its association with a range of diseases (1); however, despite various effects being reported in a range of tissues, its precise physiological function remains unknown. GPR35 has previously been shown to couple to $G\alpha_{13}$ and β-arrestin-2 (2), and is thought to couple to $G\alpha_{i/o}$ (especially in the nervous system) due to reported pertussis toxin-sensitive effects of some ligands (3). Thus, GPR35 might display G protein-dependent signalling bias in a ligand-or tissue-specific manner. In addition, phosphorylation may be involved in tissue-specific G protein versus β-arrestin bias. Since a detailed understanding of GPR35 signalling mediators is vital in the search for a function, we aimed to characterise the molecular coupling and phosphorylation profile of the receptor in order to guide physiological studies into GPR35 signalling and function.

Using a novel intramolecular bioluminescence resonance energy transfer (BRET) biosensor (based on that reported by Malik et al. (4) but with the fluorescence donor mCerulean replaced with the luminescence donor Nano luciferase), we have demonstrated that GPR35 preferentially adopts a $G\alpha_{12/13}$ coupled active conformation in HEK293 cells. When stimulated with 0.1-300 μM of the reference agonist zaprinast, the maximal $G\alpha_{13}$ biosensor response was 3-fold greater than that of the $G\alpha_{12}$ sensor, and 10-, 16- and 17-fold greater than the $G\alpha_{oA}$ ($P<0.01$), $G\alpha_{i1/2}$ ($P<0.001$) and $G\alpha_{i3}$ ($P<0.001$) sensors, respectively (pooled from $n = 3$). This pattern of relative response to the $G\alpha_{13}$, $G\alpha_{12}$ and $G\alpha_{i/o}$ biosensors was observed for all other agonists tested, suggesting no ligand bias in G protein recruitment in HEK293 cells.

As well as characterising G protein recruitment, we have determined the phosphorylation profile of GPR35. Using [^{32}P] labelling of HEK293 cells stably expressing HA-tagged GPR35, we have demonstrated that GPR35 undergoes agonist-dependent phosphorylation. We observed a 4-fold increase in [^{32}P] incorporation into HA-immunoprecipitated GPR35 in response to zaprinast, which was inhibited by the antagonist CID-2745687, and a 2- to 2.5-fold increase in response to known partial agonists ($P<0.001$, $n = 3$). Using LC-MS/MS, we identified five residues in the C-terminal tail that were phosphorylated in response to maximal stimulation with 100 μM zaprinast. In HEK cells transiently expressing eYFP-tagged GPR35 and *Renilla* luciferase-tagged β-arrestin-2, single-residue substitutions of these residues to alanine resulted in differential effects on β-arrestin-2 recruitment, decreasing the maximal response to between 26% and 96% of that of the wild-type receptor. This effect was significant ($P<0.05$, pooled from $n = 3$) for four out of the five sites. Substitution of all five residues completely abolished β-arrestin-2 recruitment, creating a 'phosphorylation-negative' receptor.

These findings demonstrate a novel method for assessing G protein coupling affinity to GPR35, and suggest preferential, unbiased coupling to both $G\alpha_{13}$ and β-arrestin-2 in HEK293 cells. Utilising this biosensor in other cell lines may reveal tissue-specific coupling profiles. We have shown that β-arrestin-2 recruitment is dependent on phosphorylation of four residues in the GPR35 C-terminal tail. The phosphorylation-negative mutant generated in this study will be used to probe G protein- versus β-arrestin-dependent effects in physiological systems.

(1) Divorty *et al.* (2015) *Front Pharmacol* **6**:41.
(2) Jenkins *et al.* (2011) *Br J Pharmacol* **162**: 733–748.
(3) Guo *et al.* (2008) *J Pharmacol Exp Ther* **324**:342–351.
(4) Malik *et al*.(2013) *J Biol Chem* **288(24)**:17167–17178.