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Agonist and inverse agonist pharmacology revealed by quantitative assessment of ghrelin receptor internalisation

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The G_q protein coupled receptor for the stomach hormone, ghrelin, displays constitutive activity (Holst *et al.*, 2006). Basal ghrelin receptor signalling and constitutive internalisation can be inhibited by inverse agonists, such as [D-Arg1, DPhe5, DTrp7, 9, Leu11] substance P (SP-A), which have therapeutic potential as appetite inhibitors (Bennett *et al.*, 2009; Holliday *et al.*, 2007; Holst *et al.*, 2006). Here we study the effects of ghrelin, SP-A and the synthetic agonist GHRP-6 (His-DTrp-Ala-Trp-DPhe-Lys-$CONH_2$; Bennett *et al.*, 2009) using automated imaging and analysis of ghrelin receptor endocytosis. We also identify a fluorescent agonist for the GhrelinR, Rhodamine-GHRP-6.

The human GhrelinR cDNA (also called GHSR-1a) in pcDNA4TO (Invitrogen) was modified to include an N-terminal signal sequence and SNAP-tag (New England Biolabs). GhrelinR expression was induced in stably transfected HEK293TR cells by tetracycline (18 h, 100 ng / ml), on poly D-lysine coated 96 well plates. G_q-coupled intracellular calcium responses (at 10-20 s peak after ligand addition, 37°C) were measured using Fluo4 in HBS / 0.1 % BSA (May *et al.*, 2010), and confocal microscopy of RhoGHRP-6 and GhrelinR in living cells was performed as described (Holliday *et al.*, 2007). For platereader imaging, surface SNAP-tagged GhrelinR was labelled with SNAPsurface BG-AF488 (NEB; 0.1 µM), before cells were treated for 30 min with vehicle or ligand, including 5 µg ml^{-1} transferrin-AF633. After fixation plates were imaged (MDC IX Ultra), and analysis quantified GhrelinR fluorescence in internal transferrin containing compartments. Concentration response curves from pooled triplicate data were normalised and scaled to basal (0%) versus1 µM ghrelin (100%) responses (Prism v5.02).

Ghrelin stimulated intracellular calcium mobilisation (pEC_{50} 7.9±0.2, *n*=6) and GhrelinR internalisation (pEC_{50} 7.8±0.1, *n*=10) in tetracycline-induced HEK293 GhrelinR cells, with approximately equivalent potencies. GHRP-6 was a more potent full agonist than ghrelin for both calcium signalling (pEC_{50} 8.7±0.3, 1 µM response 131±22 % 1 µM ghrelin, *n*=3) and receptor endocytosis (pEC_{50} 8.4±0.1, 1 µM response 109±7 %, *n*=4). SP-A addition elicited only small rapid decreases in basal calcium levels measured by Fluo4 fluorescence (1 µM -27±8 %, *n*=5). However SP-A produced clear concentration-dependent decreases in the extent of basal ghrelinR internalisation (pIC_{50} 7.3±0.2, 1 µM effect -80±15 %, *n*=5). The fluorescent analogue RhoGHRP-6 was a GhrelinR agonist (calcium response at 100 nM, 68±11 % of 1 µM ghrelin, *n*=3). 100 nM RhoGHRP-6 bound to the plasma membrane of living HEK293 GhrelinR cells only after tetracycline induction, and initially co-internalised with SNAP fluorophore labelled GhrelinRs (*n*=3+). Distinct internal compartments selectively containing fluorescent ligand or receptor became evident within 15 min of agonist treatment. Thus quantitative measurement of GhrelinR internalisation, in platereader format, allows derivation of both agonist and inverse agonist potencies for the GhrelinR and provides a potential assay in screening for novel GhrelinR inverse agonists.

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