Examining the contribution of ghrelin receptor constitutive activity to different signalling pathways

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The stomach peptide ghrelin stimulates appetite via the G-protein coupled ghrelin receptor (ghrelinR /GHSR1a), which has become a therapeutic target for obesity[1]. The ghrelinR can couple to multiple G proteins as well as G-protein independent β-arrestin pathways, and displays an unusually high level of constitutive activity[2]. To explore whether the extent of spontaneous ghrelinR activation depends on the response measured, we have compared signalling profiles of the wild type (WT) ghrelinR and two non-constitutively active ghrelinR mutants (F221A and F279N[2,3]) using G-protein coupled calcium mobilisation, G-protein independent SNAP-tagged receptor internalisation and nuclear ERK translocation. All data are expressed as means ± s.e.m. for at least three independent experiments.

Calcium mobilisation, in HEK293 cells stably transfected with SNAP-tagged ghrelinR cDNAs, was assessed by measuring peak changes in Fluo4 fluorescence on an MDC FlexStation. In this assay ghrelin (pEC_{50} 8.28 ± 0.22), L692585 (pEC_{50} 8.59 ± 0.13) and GHRP-6 (pEC_{50} 8.74 ± 0.09) were equipotent agonists (n = 3). The inverse agonist [$_{D}$-Arg^{1}, $_{D}$-Phe^{5}, $_{D}$-Trp^{7,9}, Leu^{11}] substance P (SP-A) decreased basal Fluo4 fluorescence at 1 µM. F221A and F279N mutations prevented the inverse agonist response in this assay, and also increased the maximum response to agonists (e.g. fold increase over basal with ghrelin: WT 2.5 ± 0.3; F221A 15.4 ± 3.6*; F279N 11.3 ± 3.8; n = 4-6, * p < 0.05, one-way ANOVA).

Internalisation of SNAP-tagged ghrelin receptors was assessed using a confocal plate reader (IX Ultra) combined with automated image analysis[4]. Ghrelin, GHRP-6 and L692585 stimulated WT ghrelinR internalisation (30 min. stimulation), with respective pEC_{50} values of 8.10 ± 0.24, 8.30 ± 0.51 and 8.66 ± 0.10 (n = 3-5). In contrast SP-A decreased constitutive ghrelinR internalisation with a pIC_{50} of 7.25 ± 0.12 (n = 7). As observed for calcium responses, F221A and F279N mutations prevented basal internalisation and abolished the effect of SP-A, but did not alter ghrelin potency (pEC_{50} 8.07 ± 0.15 and 7.73 ± 0.24 for F221A and F279N, respectively).

Finally, the ability of ghrelin to stimulate ERK nuclear translocation was assessed using single cell confocal microscopy in dual SNAP-ghrelinR ERK2-GFP stable transfected cells. Following overnight serum starvation, stimulation of the WT ghrelinR with ghrelin (1µM) caused nuclear accumulation of ERK-GFP within 4 minutes resulting in approx. 1.4 fold increase in nuclear fluorescence (n ≥ 10 cells). F221A and F279N mutations did not prevent ghrelin stimulated nuclear ERK translocation via these receptors in a similar manner to WT (n ≥ 10 cells).

Thus the F221A and F279N mutations selectively reduce ghrelinR constitutive activity and reveal its contribution to both G protein dependent (calcium signalling) and G protein independent (internalisation) responses investigated in this study.

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