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Genetic and biased agonist-mediated reductions in β-arrestin recruitment prolong cAMP signaling at glucagon family receptors

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Receptors for the peptide hormones glucagon-like peptide-1 (GLP-1R), glucose-dependent insulinotropic polypeptide (GIPR), and glucagon (GCGR) are important regulators of insulin secretion and energy metabolism. GLP-1R agonists have been successfully deployed for the treatment of type 2 diabetes, but it has been suggested that their efficacy is limited by target receptor desensitization and downregulation due to recruitment of β-arrestins. Indeed, recently described GLP-1R agonists with reduced β-arrestin-2 recruitment have delivered promising results in preclinical and clinical studies. We therefore aimed to determine if the same phenomenon could apply to the closely related GIPR and GCGR. In HEK293 cells depleted of both β-arrestin isoforms the duration of G protein–dependent cAMP/PKA signaling was increased in response to the endogenous ligand for each receptor. Moreover, in wildtype cells, "biased" GLP-1, GCG, and GIP analogs with selective reductions in β-arrestin-2 recruitment led to reduced receptor endocytosis and increased insulin secretion over a prolonged stimulation period, although the latter effect was only seen at high agonist concentrations. Biased GCG analogs increased the duration of cAMP signaling, but this did not lead to increased glucose output from hepatocytes. Our study provides a rationale for the development of GLP-1R, GIPR, and GCGR agonists with reduced β-arrestin recruitment, but further work is needed to maximally exploit this strategy for therapeutic purposes.

The receptors for the glucagon-like peptide-1 (GLP-1R), glucose-dependent insulinotropic polypeptide (GIPR), and glucagon (GCGR) are major pharmacological targets in metabolic diseases such as type 2 diabetes (T2D) and obesity (1). Each of these receptors is present on pancreatic beta cells, and an important component of their overall metabolic actions when physiologically or pharmacologically activated is augmentation of glucose-stimulated insulin release (2, 3). In hepatocytes, GCGR facilitates glucose output, which may be undesirable in T2D; however, its "energy wasting" effect in peripheral tissues (2) could mitigate hyperglycemia by weight loss and associated improvements in insulin sensitivity.

GLP-1R, GIPR, and GCGR are closely related G protein–coupled receptors (GPCRs) of the class B (secretin) family. When activated, they engage the G protein $G\alpha_s$, which in beta cells is coupled to insulin secretion *via* generation of cyclic adenosine monophosphate (cAMP) (4), and β-arrestins, which diminish G protein signaling by steric hindrance (5) and, according to some (6) but not all (7) reports, can initiate non–G protein signaling cascades such as phosphorylation of mitogen-activated protein kinases (MAPKs). To varying degrees, each of these receptors undergoes agonist-mediated endocytosis (8), which fine tunes the spatial origin and duration of their intracellular signaling responses (9, 10).

The balance between recruitment and activation of intracellular signaling effectors and subsequent receptor trafficking can be ligand-specific—a pharmacological concept known as "biased agonism" (11). A number of examples of bias at the GLP-1R have been described, including both naturally occurring (12, 13) and pharmacological (14, 15) orthosteric agonists. Importantly, G protein-biased GLP-1R agonists derived from exendin-4 lead to increases in sustained insulin secretion through avoidance of GLP-1R desensitization, reduction of GLP-1R endocytosis, and resultant attenuation of GLP-1R downregulation over pharmacologically relevant time periods (16, 17). Moreover, a GLP-1R/GIPR dual agonist (Tirzepatide) with promising results for the treatment of T2D in clinical

This article contains supporting information.

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trials (18) has recently been reported to show pronounced G protein bias at the GLP-1R, although not at the GIPR (19). In view of the current drive to develop incretin analogs jointly targeting GLP-1R, GCGR, and GIPR (1), we sought to establish whether biased agonism could similarly be achieved at the latter two receptors and to determine if this is associated with prolonged signaling responses, as seen with the GLP-1R.

In this work, we first compare β-arrestin recruitment and activation profiles of GLP-1R, GIPR, and GCGR activated by their cognate ligands and subsequently demonstrate how the absence of β-arrestins affects patterns of intracellular signaling and trafficking. We also find that a number of substitutions close to the N terminus of the cognate ligand for each receptor result in reductions in both cAMP signaling and β-arrestin-2 recruitment, with quantifiable bias in favor of cAMP in some cases. However, compared with our previous study with biased exendin-4 analogs at the GLP-1R (16), the degree of bias achieved here was more modest. Moreover, although bias-related differences were apparent in downstream responses such as insulin secretion, these only occurred at high agonist concentrations.

Results

Coupling of GLP-1R, GIPR, and GCGR to intracellular effectors and endocytosis

We first performed studies to compare responses to the cognate agonist for each receptor in HEK293T cells. Specifically, for GLP-1R we used GLP-1(7–36)NH_2, for GIPR we used GIP(1–42), and for GCGR we used full–length GCG(1–29). These ligands are referred to henceforth as GLP-1, GIP, and glucagon (GCG). Using NanoBiT complementation (20) to detect ligand-induced recruitment of LgBiT-tagged mini-G proteins (21) to each of the receptors tagged at the C terminus with the complementary SmBiT sequence, we confirmed a robust ligand-induced mini-G_s response, but more minor increases with mini-G_q and mini-G_i (Fig. 1*A*, Fig. S1*A*). This is in keeping with the consensus that glucagon family receptors are primarily coupled to cAMP signaling *via* $G\alpha_s$, with system-dependent engagement with other Gα subtypes under some circumstances (22, 23). Moreover, LgBiT-β-arrestin-2 recruitment responses could be detected in all cases but were more transient than for mini-G proteins, matching the pattern seen with pharmacological GLP-1R agonists (24). Notable differences between receptor types included (1) substantially greater amplitude for mini-G_s recruitment for GLP-1R than for GCGR and GIPR, with the latter also showing slower kinetics ($t_{1/2}$ = 7.1 ± 0.4 min *versus* 1.5 ± 0.2 min for GLP-1R, $p < 0.05$ by unpaired *t* test); (2) mini-G_i and mini-G_q responses were virtually undetectable for GIPR; and (3) markedly reduced recruitment of β-arrestin-2 to GIPR compared with GLP-1R and GCGR, in keeping with another report (25). These and other responses are quantified from the area under the curve (AUC) in Figure 1*G* as well as Fig. S1*A*.

After recruitment to activated GPCRs, β-arrestins undergo conformational rearrangements that are important for their functions (26, 27). Using a recently developed intramolecular BRET-based biosensor (28) we compared the ability of GLP-1R, GIPR, and GCGR to activate β-arrestin-2 when stimulated by their cognate agonists. Here, comparable ligand-induced BRET signals were detected with each receptor (Fig. 1*B*, Fig. S1*B*), highlighting how measuring recruitment of intracellular effectors *per se* may not provide the full picture for how a receptor or ligand can engage different intracellular pathways. Note that the BRET ratio obtained in the presence of each transfected receptor prior to stimulation was identical, arguing against receptor-specific differences in constitutive β-arrestin-2 activation (Fig. S1*B*).

β-Arrestin recruitment is classically linked to GPCR endocytosis (29), although conflicting evidence exists for its role in controlling trafficking of incretin receptors (30–34). We first used diffusion-enhanced resonance energy transfer (DERET) (35) to monitor agonist-induced loss of surface labeled SNAP-tagged receptors transiently expressed in HEK293T cells. Robust internalization was noted for GLP-1R, whereas GIPR internalization was less extensive, and no ligand-induced change in DERET signal could be detected for GCGR (Fig. 1*C*, Fig. S1*C*). Endocytic profiles were confirmed using an alternative approach based on reversible SNAP-tag labeling, in which the fluorescent probe BG-S-S-649 is cleaved from residual surface receptors after agonist-induced internalization using the cell-impermeant reducing agent Mesna (17, 36). A time-course study showed rapid and extensive loss of surface SNAP-GLP-1R after GLP-1 treatment, whereas internalization of the other two class B GPCRs was more limited (GIPR) or virtually absent (GCGR) (Fig. 1*D*, Fig. S1*D*). Examples of the effect of Mesna cleavage are shown in Figure 1*E*, and higher-resolution images showing ligand-induced distribution changes of surface-labeled SNAP-GLP-1R, -GIPR, and -GCGR are shown in Figure 1*F*.

On comparison of the measured responses for each receptor with GLP-1R as the reference, two notable observations were that (1) the largest-amplitude responses were seen with GLP-1R for all readouts, and (2) GCGR showed a greater ligand-induced recruitment of β-arrestin-2 (and other effectors) than GIPR, yet this did not translate to a corresponding increase in ligand-induced endocytosis (Fig. 1*G*).

Effect of β-arrestin depletion on GLP-1R, GIPR, and GCGR signaling and trafficking

To further investigate the role of β-arrestins in incretin receptor behaviors we used HEK293 cells in which both β-arrestins were deleted by CRISPR-Cas9 (37). First, cAMP signaling responses to each cognate ligand were compared in wildtype or β-arrestin-knockout cells transiently transfected with the relevant SNAP-tagged receptor. In wildtype cells, a robust cAMP response was observed after 10-min stimulation but was substantially attenuated after 60 min (Fig. 2*A*, Table 1). In contrast, the reduction in efficacy over time was much less marked in β-arrestin-knockout cells, suggesting that β-arrestins do, as expected, contribute to the attenuation of $G\alpha_s$-mediated responses for GLP-1R, GIPR, and GCGR. We also used the FRET biosensor AKAR4-NES (38) to detect

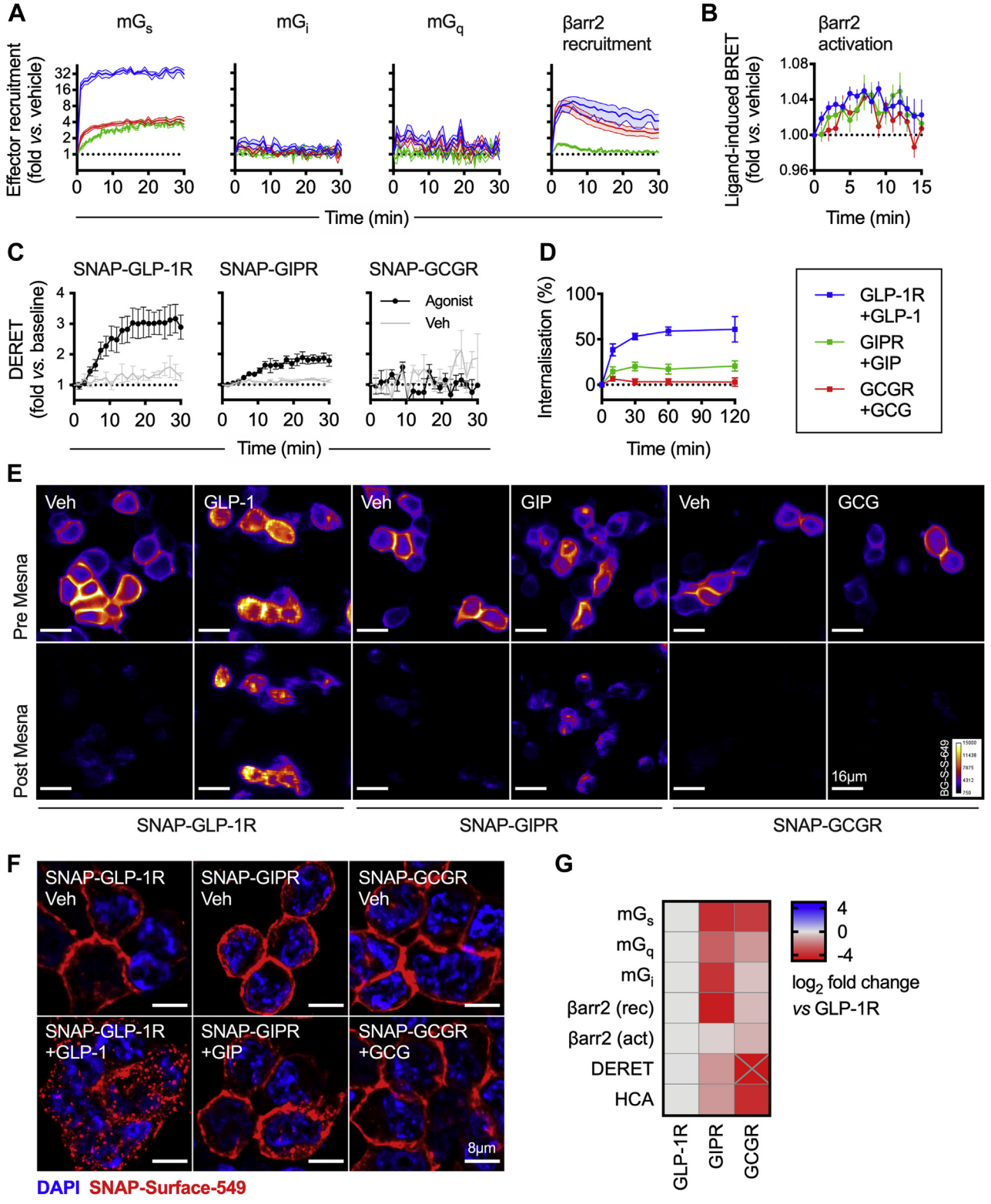

Figure 1. Signaling and internalization properties of GLP-1R, GIPR, GCGR. *A*, mini-G_s, -G_i, -G_q, and β-arrestin-2 recruitment responses in HEK293T cells transiently transfected with each SmBiT-tagged receptor and LgBiT-tagged effector and stimulated with 100 nM GLP-1, GIP, GCG or vehicle; $n = 4$ for GLP-1R and GCGR and $n = 5$ for GIPR. *B*, β-Arrestin-2 activation response in HEK293T cells transiently transfected with each receptor and NLuc-4myc-βarr2-CyOFP1 and stimulated with 100 nM GLP-1, GIP, GCG or vehicle, $n = 6$. *C*, internalization of each SNAP-tagged receptor in HEK293T cells stimulated with 100 nM GLP-1, GIP, GCG, or vehicle, measured by DERET, $n = 4$. *D*, time course showing internalization of each SNAP-tagged receptor in response to 100 nM GLP-1, GIP, or GCG, measured by reversible SNAP-tag labeling in transiently transfected HEK293T cells, $n = 5$. *E*, representative images of transiently transfected HEK293T cells expressing each SNAP-tagged receptor and labeled with BG-S-S-649 prior to treatment ±100 nM GLP-1, GIP, or GCG. Images were acquired before and after removal of residual surface BG-S-S-649 using Mesna; the scale bar represents 16 μm. *F*, representative high-resolution images from $n = 2$ experiments of HEK293T cells transiently expressing each SNAP-tagged receptor and labeled with SNAP-Surface-549 before treatment ±100 nM GLP-1, GIP, or GCG; the scale bar represents 8 μm. *G*, heatmap summary of signaling and internalization responses shown in this figure, normalized to the GLP-1R

cytoplasmic protein kinase A (PKA) activation in each cell type (Fig. 2*A*). Plate-reader measurements at multiple agonist concentrations allowed construction of dose-response curves from the overall AUC (Fig. 2*B*, Table 2), demonstrating that potency for PKA activation was increased in β-arrestin-knockout cells. This suggests that an increased cAMP signaling in the absence of β-arrestins is also propagated to downstream targets.

β-Arrestins have been observed on many occasions to facilitate signaling by MAPKs such as ERK1/2 (39), and this pathway has been implicated in GLP-1R action in beta cells (31, 40). Interestingly, the peak of ERK1/2 phosphorylation observed after 5 min of ligand stimulation was preserved in β-arrestin knockout cells, but the duration of the phosphorylation response was reduced for GLP-1R and GCGR, although not for GIPR (Fig. 2*C*). These results are in keeping with newer reports for other GPCRs indicating that the presence of β-arrestins is not essential for ERK1/2 signaling *per se* (7, 37) but may be required for sustained ERK1/2 phosphorylation (40).

To determine the β-arrestin dependency of incretin receptor endocytosis, we performed further high content microscopy internalization studies. Surface expression of each receptor was similar in each cell type (Fig. 2*D*). After a 30-min stimulation period, a modest numerical reduction in agonist-induced GLP-1R and GIPR internalization was noted in β-arrestin knockout cells compared with the wildtype, but this was only statistically significant for GIPR. No internalization of GCGR was detected, as expected. Thus, these experiments corroborate our earlier observations that the absence of β-arrestins has a relatively minor effect on GLP-1R endocytosis (16, 32), with a partial effect seen also with GIPR.

Thus, these results provide initial evidence that β-arrestin recruitment controls both duration and amplitude of cAMP signaling by glucagon family receptors but is not essential for transient ERK1/2 signaling responses or endocytosis.

Effects of N-terminal region mutations to GLP-1, GIP, and GCG on signaling and trafficking responses

Biased agonism, in which certain receptor conformations are preferentially stabilized to facilitate engagement with specific intracellular effectors, may provide a means to selectively increase therapeutic efficacy (41). As the ligand N-terminal region plays a key role in the activation of class B GPCRs (42) and is linked to GLP-1R biased agonism (16), we introduced single amino acid substitutions at or close to the N termini of each endogenous ligand (Table 3 for full amino acid sequences) and tested for intracellular cAMP production (Fig. 3*A*) and β-arrestin-2 recruitment (Fig. 3*B*). For each receptor target, all N-terminally modified analogs retained full efficacy for cAMP, but with reduced potency (full parameter estimates are given in Table S1). Potency for β-arrestin-2 recruitment was also reduced, and in the majority of cases, a reduction in efficacy was also observed. Transduction ratios (43) were calculated to quantify the relative signaling or recruitment impact of each N-terminal region substitution in each ligand for each response (Fig. 3, *C*–*D*). Chiral substitution of the first amino acid to dHis1 (GLP-1, GCG) or dTyr1 (GIP), as well as Gly2 and dGln3, were less well tolerated by GIP and GCG than by GLP-1; for example, GIP-dGln3 showed a 100-fold reduction in cAMP potency compared with wildtype GIP, *versus* a 10-fold reduction seen for GLP-1-dGln3 compared with GLP-1. Moreover, comparison of the relative impact of each substitution on cAMP *versus* β-arrestin-2 responses showed that all compounds tested exhibited at least a trend for bias in favor of G protein–dependent cAMP production, albeit not statistically significant (as indicated by 95% confidence intervals crossing zero) in some cases (Fig. 3*E*). The large error bars for the bias estimate for GIP-dGln3 reflect the limitations of this method for bias calculation for extremely weak partial agonists (44).

We also compared GLP-1R and GIPR internalization induced by these N-terminally substituted agonists and found that that in all cases receptor internalization was reduced when measured by high content microscopy (Fig. 3*F*) or DERET (Fig. 3*G*) in CHO-K1 cells stably expressing SNAP-GLP-1R or SNAP-GIPR. The GCG analogs were not studied with this assay as GCG itself was without effect.

Overall, these results highlight how the N termini of each ligand play important roles in receptor activation and initiation of endocytosis. It should be noted that the magnitude of response bias with the GLP-1 analogs tested here is smaller than for exendin-4-derived biased GLP-1R agonists, a finding that is consistent with our recent exploration of GLP-1/exendin-4 chimeric peptides (17).

Biased agonist responses in a beta cell and hepatocyte context

As pancreatic beta cells are a target for GLP-1, GIP, and GCG (45), we investigated whether the pharmacological characteristics of N-terminally modified agonists described above could enhance insulin secretion, as previously demonstrated for biased exendin-4-derived GLP-1RAs (16). We used incretin-responsive rat insulinoma-derived INS-1 832/3 cells (46), in which we first confirmed expression of GLP-1R, GIPR, and GCGR by qPCR (Fig. 4*A*). Using N-terminally substituted GLP-1 and GIP analogs, we found a subtly increased maximal sustained insulin secretion with a number of analogs compared with their respective parent ligand, but this was only apparent at concentrations above 1 μM (Fig. 4, *B*–*C*, Table S2). The prolonged incubation in these experiments was specifically selected with the aim of better replicating the *in vivo* situation, where therapeutic ligands with extended pharmacokinetics lead to a state of continuous receptor activation. The majority of analogs displayed reduced potency for insulin

response and expressed as a $\log_2$ fold change. β-Arrestin-2 recruitment ("rec," Fig. 1*A*) and activation ("act," Fig. 1*B*) responses are both shown. GCGR DERET response falls below the displayed range and is marked with "X." See also Fig. S1. Data are represented as mean ± SEM. DERET, diffusion-enhanced resonance energy transfer; GCG, glucagon; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor.

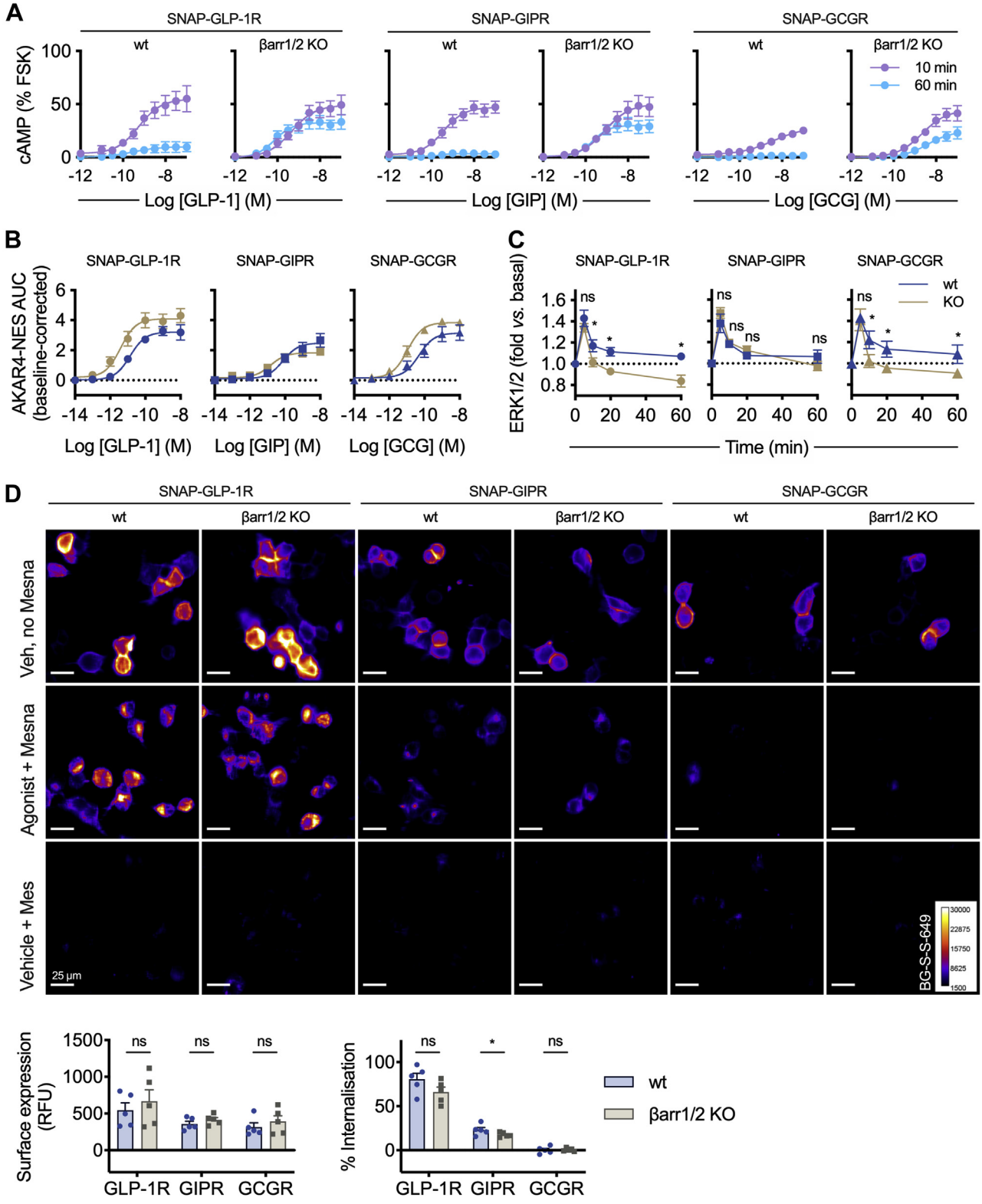

Figure 2. GLP-1R, GIPR, and GCGR responses in the absence of β-arrestins. *A*, cAMP responses at 10 or 60 min to GLP-1, GIP, or GCG, in wildtype (wt) or dual β-arrestin knockout (βarr1/2 KO) HEK293 cells transiently transfected with the indicated SNAP-tagged receptor, normalized to forskolin (FSK, 10 μM) response, respectively, $n = 5$, 4-parameter fits shown. *B*, dose responses for PKA activation in wildtype or dual β-arrestin knockout HEK293 cells transiently cotransfected with each SNAP-tagged receptor and AKAR4-NES, calculated from FRET signal AUC and normalized to vehicle responses, three-parameter fits shown, $n = 5$. See also Fig. S2. *C*, time course for ERK1/2 phosphorylation in wildtype or dual β-arrestin knockout HEK293 cells transiently transfected with each SNAP-tagged receptor and stimulated with 100 nM GLP-1, GIP or GCG, normalized to basal response. Time points compared by randomized block two-way ANOVA with Sidak's test, $n = 6$. *D*, representative images showing internalization of each transiently transfected SNAP-tagged receptors in wildtype or dual β-arrestin knockout HEK293 cells, labeled with BG-S-S-649 prior to 100 nM agonist (or vehicle) treatment, with removal of residual surface receptor using Mesna where indicated; the scale bar represents 25 μm. Surface expression and ligand-induced internalization are quantified from $n = 5$ experiments, with comparisons by paired *t* tests. $^*p < 0.05$ by statistical test indicated in the text. Data are represented as mean ± SEM with individual replicates in some places. GCG, glucagon; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor.

Table 1
Effect of β-arrestin deletion on agonist-induced cAMP responses in HEK293 cells

	Wildtype		β-Arrestin-1/2 KO	
	10 min	60 min	10 min	60 min
SNAP-GLP-1R + GLP-1				
E_{max} (%FSK)	54 ± 10	10 ± 5[a]	50 ± 10	54 ± 6
Log EC_{50} (M)	−9.3 ± 0.1	−9.3 ± 0.1	−9.6 ± 0.1	−10.4 ± 0.2[a]
Hill slope	±0.3	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.2
SNAP-GIPR + GIP				
E_{max} (%FSK)	47 ± 6	4 ± 0[a]	51 ± 10	30 ± 6[a]
Log EC_{50} (M)	−9.4 ± 0.1	−9.6 ± 0.1	−9.1 ± 0.1	−9.7 ± 0.1[a]
Hill slope	0.9 ± 0.0	1.6 ± 0.1[a]	0.7 ± 0.1	1.2 ± 0.1[a]
SNAP-GCGR + GCG				
E_{max} (%FSK)	24 ± 2	2 ± 0[a]	48 ± 9	25 ± 8[a]
Log EC_{50} (M)	−8.7 ± 0.2	−8.1 ± 0.3	−8.6 ± 0.2	−8.5 ± 0.2
Hill slope	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.0

Mean parameter estimates ±SEM from responses depicted in Figure 2*A*, $n = 5$.
GCG, glucagon; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor.
[a] $p < 0.05$ by two-way randomized block ANOVA with Sidak's test comparing 10 *versus* 60 min.

secretion, as they had for acute cAMP production in the signaling assays presented in Figure 3. For GIP analogs, maximal insulin secretion was inversely correlated with maximal β-arrestin-2 recruitment, whereas for GLP-1 analogs the relationship was less clear, and in both cases the regression line was shallow (Fig. 4*D*). As GCG can cross-react with GLP-1R in beta cells (47), we tested each N-terminally modified GCG analog in both wildtype INS-1 832/3 cells and a subclone in which GLP-1R had been knocked out by CRISPR-Cas9 (48). This showed that the overall response was dominated by GLP-1R-dependent high-dose effects absent in GLP-1R knockout cells, with no clear GCGR-dependent advantageous effect for any analog (Fig. 4*E*, Table S2).

GCG stimulates glycogenolysis and gluconeogenesis in hepatocytes. Recently, ablation of β-arrestin-2 in hepatocytes was found to increase hepatic glucose output in response to GCG (33). We used Huh7 cells stably expressing GCGR ("Huh7-GCGR") (49) to assess responses to prolonged stimulation with biased GCG analogs, to determine if differences in β-arrestin recruitment could affect sustained GCGR signaling in a hepatocyte context. Comparisons of maximum cAMP accumulation after 16-h stimulation with each ligand revealed

Table 2
Effect of β-arrestin deletion on agonist-induced cytoplasmic PKA activation in HEK293 cells

	Wildtype	β-Arrestin-1/2 KO
SNAP-GLP-1R + GLP-1		
E_{max} (baseline-corrected AUC)	3.2 ± 0.4	4.1 ± 0.5
Log EC_{50} (M)	−10.9 ± 0.1	−11.5 ± 0.3[a]
SNAP-GIPR + GIP		
E_{max} (baseline-corrected AUC)	2.5 ± 0.5	1.8 ± 0.2
Log EC_{50} (M)	−10.1 ± 0.0	−10.9 ± 0.1[a]
SNAP-GCGR + GCG		
E_{max} (baseline-corrected AUC)	3.1 ± 0.5	3.9 ± 0.2
Log EC_{50} (M)	−10.3 ± 0.1	−11.0 ± 0.2[a]

Mean parameter estimates ±SEM from responses depicted in Figure 2*B*, $n = 5$.
GCG, glucagon; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor.
[a] $p < 0.05$ by paired *t* test comparing wildtype *versus* knockout.

that a greater efficacy for cAMP production was achieved by ligands with reduced β-arrestin-2 recruitment (Fig. 4*F*, Table S2), and in the case of the -Phe1 and -dGln3 compounds, cAMP potency was also greater than for GCG, despite being at least 10-fold less potent acutely (see Fig. 3). However, this did not translate to differential changes in GCGR-induced upregulation of the gluconeogenic enzyme glucose-6-phosphatase (Fig. 4*G*) nor were any significant differences seen for the production of glucose in primary mouse hepatocytes (Fig. 4*H*).

Overall, these results indicate that analogs of GLP-1 and GIP with reduced β-arrestin-2 recruitment can augment glucose-stimulated insulin secretion, but with the peptides evaluated in this study, this effect was only apparent at high agonist concentrations. For GCG analogs, prolonged cAMP signaling was seen with agonists displaying reduced β-arrestin-2 recruitment, but this did not translate to increases in downstream responses linked to hyperglycemia in the models tested.

Discussion

This study builds on our earlier work using biased GLP-1R agonists derived from exendin-4 and GLP-1 bearing amino acid substitutions close to the N terminus, in which we also demonstrated prolonged GLP-1R signaling in the absence of β-arrestins but a minor effect of these on endocytosis (16, 17, 32). In the present work we extend these observations to GIPR and GCGR, members of the same class B GPCR family and major investigational targets for metabolic disease. The key findings of this study are: (1) the absence of β-arrestins facilitates prolonged cAMP/PKA signaling with each receptor, with either nonsignificant or partial effects on endocytosis, and (2) amino acid substitutions at or close to the N termini of GLP-1, GIP ,and GCG can diminish β-arrestin-2 recruitment efficacy, with a somewhat lesser effect on cAMP signaling, but the degree of effector selectivity is reduced compared with what we have previously observed with exendin-4 analogs at the GLP-1R (16), and the impact on prolonged insulin secretion in pancreatic beta cells is more limited.

As expected, GLP-1R, GIPR, and GCGR were able to recruit mini-G_s and β-arrestin-2 when stimulated with a high concentration of their cognate agonist. However, GIPR responses were in general of reduced amplitude, matching previous observations (25). Interestingly, β-arrestin-2 activation measured by a conformational BRET-based biosensor (28) appeared similar for each receptor, in spite of the difference in recruitment. As proximity-based techniques such as nanoBiT complementation are susceptible to distance constraints imposed by the conformation of the target protein, it is plausible that GIPR-induced β-arrestin-2 recruitment is actually higher than suggested by our results. This might be resolved using a target-agnostic technique such as bystander BRET (50) to monitor β-arrestin recruitment to the plasma membrane, although apparent differences in the segregation of GIPR and GLP-1R into liquid ordered *versus* liquid disordered nanodomains (32) may lead to further confounding depending on acceptor localization. Further possibilities that could

Table 3
Amino acid sequences of ligands in this study

Ligand	Sequence
GLP-1-derived ligands	
GLP-1(7–36)NH_2	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
GLP-1-dHis1	(dH)AEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
GLP-1-Phe1	FAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
GLP-1-Gly2	HGEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
GLP-1-dGln3	HA(dQ)GTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
GIP-derived ligands	
GIP(1–42)	YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
GIP-dTyr1	(dY)AEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
GIP-Phe1	FAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
GIP-Gly2	YGEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
GIP-dGln3	YA(dQ)GTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
GCG-derived ligands	
GCG(1–29)	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
GCG-dHis1	(dH)SQGTFTSDYSKYLDSRRAQDFVQWLMNT
GCG-Phe1	FSQGTFTSDYSKYLDSRRAQDFVQWLMNT
GCG-Gly2	HGQGTFTSDYSKYLDSRRAQDFVQWLMNT
GCG-dGln3	HS(dQ)GTFTSDYSKYLDSRRAQDFVQWLMNT

Sequences are given in standard single letter amino acid code, with D-histidine, D-tyrosine, and D-glutamine indicated as "dH," "dY," and "dQ," respectively.
GCG, glucagon; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1.

complicate comparisons between the activation and recruitment assays are differences in receptor-effector stoichiometry related to promoter activity and assay dynamic range.

β-Arrestin recruitment to GPCRs sterically blocks G_s signaling. Indeed, our data indicate how the absence of β-arrestins prevents a decline in cAMP production during continual agonist stimulation after an initial peak, similar to observations with the β2-adrenergic receptor (37) and adding to the evidence that β-arrestins restrain cAMP signaling at GLP-1R (16) and GCGR (33). Assuming that prolonged activation of each receptor is considered therapeutically desirable, this provides a strong rationale for developing of G protein–biased agonists capable of generating longer-lasting signaling responses. We note also that the relative augmentation of cAMP production at the later time point in β-arrestin knockout cells was similar for each receptor, in keeping with their apparently similar ability to induce β-arrestin-2 conformational change (as in Fig. 1). Contrasting with the situation for cAMP/PKA, agonist-induced ERK1/2 phosphorylation tended to be preserved at early time points in β-arrestin knockout cells but reduced at later time points. Multiple factors are implicated in incretin receptor–mediated ERK1/2 phosphorylation (42, 51, 52), and for GPCRs more widely there is ongoing controversy concerning the relative contribution of G proteins *versus* β-arrestins to MAPK activation (7, 53). Nevertheless, our data suggest that β-arrestins may indeed play a role specifically in sustained (rather than acute) ERK1/2 phosphorylation, which has been implicated in GLP-1R-induced protection against apoptosis in beta cells (40). *Via* a different mechanism, ERK1/2 is also implicated in GIPR-mediated beta cell survival (54), although we did not observe reduced GIP-induced ERK1/2 phosphorylation in β-arrestin knockout cells in our experiments. It is unclear whether potential reductions in signaling pathways engaged by ERK1/2 and other putative β-arrestin-scaffolded MAPKs are relevant to the therapeutic action of incretin receptor–targeting biased ligands. Reassuringly, there was no evidence of reduced beta cell survival in mice chronically treated with a biased GLP-1R agonist with undetectable β-arrestin recruitment (55).

Increasing emphasis is now placed on understanding ligand-specific effects on receptor trafficking owing to its potential importance in the spatiotemporal control of intracellular signaling (10). In our hands, the absence of both β-arrestin isoforms had a surprisingly small effect on GLP-1R, which is in keeping with our earlier data (16, 32). For GIPR, a partial reduction in internalization was observed, consistent with the report of Gabe *et al.*, (56) who showed a partially reduced GIPR internalization in the same cell models using DERET. Overall, both GLP-1R and GIPR can continue to undergo ligand-induced endocytosis in the absence of β-arrestins, suggesting the existence of β-arrestin-independent endocytic mechanism(s). We cannot, however, exclude the possibility of compensatory upregulation or rewiring of secondary endocytic pathways in β-arrestin knockout cells, which could disguise a more significant role for β-arrestins in the endocytosis of these receptors.

GLP-1 is more dependent on its N terminus for binding to the GLP-1R than is exendin-4 (57). Sequential truncation of the first nine amino acids of exendin-4 results in only a modest reduction in binding affinity but virtually abolishes the binding of GLP-1 (23, 58). Thus, the reduction in signaling potency resulting from an N-terminal amino acid substitution within the GLP-1 backbone may be secondary to reduced affinity, whereas the same change in exendin-4 might have little impact on occupancy, thereby allowing the modified ligand to achieve biased responses at higher potency. In agreement with this concept, acute cAMP signaling potencies for exendin-dHis1 and exendin-Phe1 in our earlier study were, respectively, no different to and 2.5-fold lower than for exendin-4 (16), whereas contrastingly in the present work, the same substitutions to the GLP-1 N terminus reduced cAMP potency by, respectively, a factor of 6 and 10. This might limit the potential for these modified GLP-1 analogs to improve downstream signaling outputs during prolonged stimulation, except at maximal doses when receptor occupancy is high, and even then,

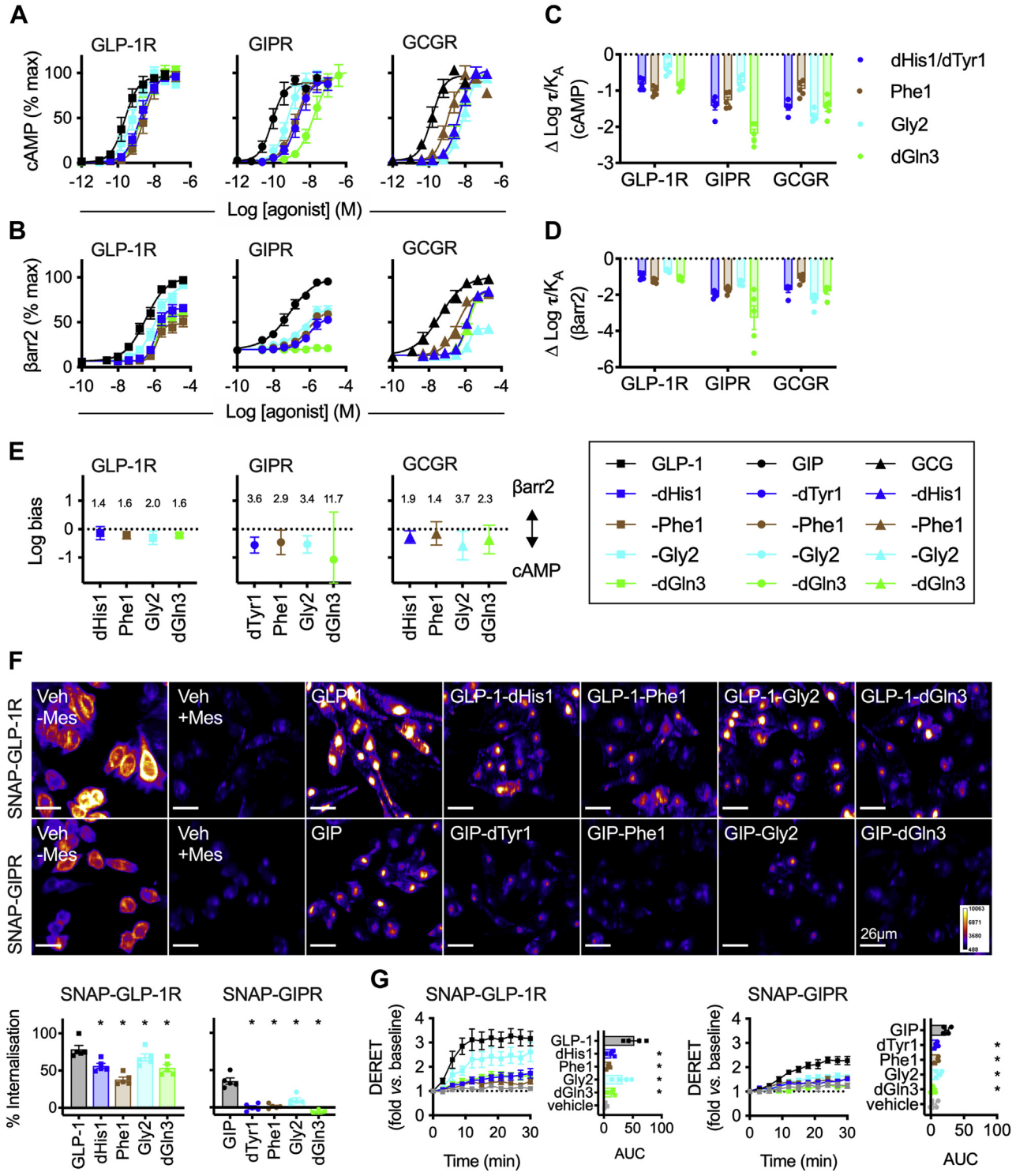

Figure 3. Biased incretin analogs with N-terminal region substitutions. *A*, cAMP responses in PathHunter CHO-GLP-1R, CHO-GIPR, or CHO-GCGR cells (as appropriate) to analogs of GLP-1, GIP, and GCG, 30-min stimulation, $n = 5$, 4-parameter fits shown. *B*, as for (*A*) but β-arrestin-2 recruitment responses. *C*, the relative impact of each amino acid substitution on cAMP signaling is shown by subtracting Log τ/K_A for the reference agonist (GLP-1, GIP, or GCG) from that of each analog on a per-assay basis. *D*, As for (*C*) but for β-arrestin-2 recruitment. *E*, biased agonism for N-terminally modified GLP-1, GIP, and GCG analogs at their cognate receptors, calculated as normalized log transduction ratios [$\Delta\Delta\log(\tau/K_A)$] relative to GLP-1, GIP, or GCG, respectively. The numerical degree of bias is indicated for each ligand after anti-log transformation. *F*, representative images showing GLP-1R and GIPR internalization after 30-min stimulation with indicated agonist at 100 nM, with quantification below from $n = 5$ experiments and comparison by one-way randomized block ANOVA with Dunnett's test *versus* GLP-1 or GIP (as appropriate); the scale bar represents 26 μm. *G*, SNAP-GLP-1R or SNAP-GIPR internalization in CHO-K1 cells treated with the indicated agonist (100 nM), measured by DERET, $n = 4$, AUC *versus* GLP-1 or GIP compared by randomized block one-way ANOVA with Dunnett's test. *$p <$ 0.05 by statistical test indicated in the text. Data are represented as mean ± SEM (with individual replicates in some cases), except for bias plots where error bars indicate 95% confidence intervals. GCG, glucagon; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor.

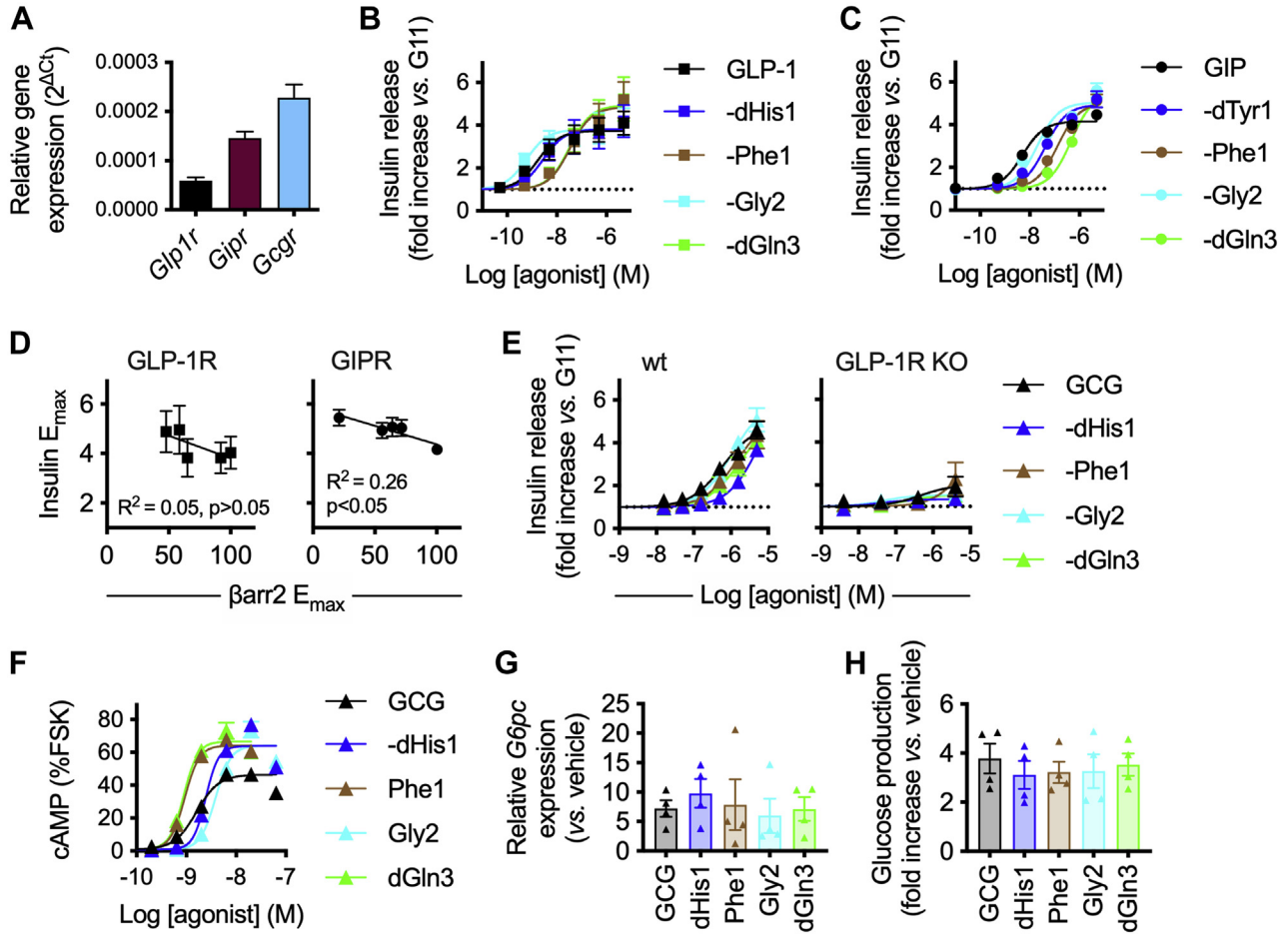

Figure 4. Responses in INS-1 832/3 cells, Huh7 cells, and primary hepatocytes. *A* expression of *Glp1r*, *Gipr*, and *Gcgr* in INS-1 832/3 cells, determined by qPCR, normalized to expression of endogenous control gene 18S by $2^{-\Delta Ct}$, $n = 2$. *B*, insulin secretory responses in INS-1 832/3 cells treated for 16 h with GLP-1 analogs at 11 mM glucose ("G11"), $n = 5$, three-parameter fits shown. *C*, As for (*B*) but with GIP analogs. *D*, correlation of GLP-1 and GIP analog maximum insulin secretion and maximum β-arrestin-2 recruitment (Fig. 3) by linear regression. *E*, insulin secretory responses in wildtype and GLP-1R KO INS-1 832/3 cells treated for 16 h with GCG analogs at 11 mM glucose, $n = 5$, three-parameter fits shown. *F*, cAMP responses to GCG analogs in Huh7-GCGR cells treated for 16 h, relative to response to forskolin (10 min, 10 μM), $n = 4$, four-parameter fits shown. *G*, effect of prolonged (16-h) exposure to indicated agonist (10 nM) on upregulation of *G6pc* in Huh7-GCGR cells, $n = 4$. *H*, effect of prolonged (16-h) exposure to indicated agonist (100 nM) on glucose production by primary mouse hepatocytes, $n = 4$, expressed as fold change to vehicle stimulation. For (*G*) and (*H*), no treatment response was significantly different from that of GCG, by one-way randomized block ANOVA with Dunnett's test. Data are represented as mean ± SEM and individual replicates in some cases. GCG, glucagon; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor.

reduced mini-G_s recruitment is clearly demonstrated with other GLP-1R agonists with N-terminal modifications at supramaximal stimulatory concentrations (17). As for GLP-1, the N-termini of both GCG and GIP are also known to play a major role in the affinity for their cognate receptors, with truncation of the terminal amino acid residue resulting in a >10-fold loss of affinity in both cases (59, 60). The resulting reductions in agonism have the potential to partly counterbalance benefits from reduced β-arrestin-mediated desensitization, partly reconciling the discrepancy between the modest increases in insulinotropic efficacy over 16 h with N-terminally modified ligands *versus* the striking differences in the duration of cAMP signaling in β-arrestin knockout cells over 60 min with nonmodified GLP-1, GCG, and GIP.

A number of analogs tested in this report have previously been described, due in part to the interest in reducing ligand sensitivity to degradation by the N-terminal targeting exopeptidase dipeptidyl dipeptidase-4 (DPP-4) (61). Published potency or affinity measures for GLP-1-dHis1 (62) and -Gly2 (63) were broadly in agreement with our results, although GLP-1-Phe1 was found to be well tolerated for cAMP signaling potency in RIN-T3 cells (64), contrasting with the deleterious effect we observed. Differences in cell type, receptor species, incubation times, and other factors may influence responses to agonists, complicating direct comparisons with reported values in the literature (65). The affinity of GIP-dTyr1 was reduced 10-fold compared with that of unmodified GIP (66), similarly to our results, whereas the -Gly2 substitution was well tolerated (67). GIP-Phe1 has been used as a GIPR I^{125}-radioligand (68). These datasets are complemented here by our measures of bias between cAMP and β-arrestin recruitment, and endocytosis, which have not previously been reported for these ligands, or indeed for any putative biased GCGR or GIPR agonist to our knowledge.

A further factor that might contribute to the relative lack of effect on downstream responses to biased GLP-1, GIP, and GCG analogs during prolonged incubations is enzymatic peptide degradation, for example, by neutral endopeptidase 24.11, found on pancreatic beta cell membranes and capable of hydrolyzing GLP-1, GCG, and, to a lesser extent, GIP (69), or endothelin converting enzyme-1 (70) situated predominantly in endosomal compartments. DPP-4, also expressed by beta cells (71), is also likely to contribute, although the modified N termini of the ligands tested may confer some resistance to its action. Sequence optimization to increase proteolytic stability during our extended *in vitro* studies may be required to maintain adequate ligand concentration to fully manifest consequences of biased agonism. In the *in vivo* setting, fatty acid conjugation such as in liraglutide (72) protects against neutral endopeptidase 24.11 and DPP-4 degradation, presumably as the resultant albumin-bound form of the ligand is inaccessible to the enzymes. One possible future approach would be to test acylated forms of the ligands described herein to determine if sustained exposure to the N-terminally substituted forms led to enhanced metabolic effects. In addition, as our beta cell studies were performed with INS-1 832/3 clonal beta cells, it would be important to validate key findings in primary islets, ideally from humans, to ensure that the observations are not an artefact of the model used. In particular, the inherent interconnection of different cell types in intact islets is lost in isolated cell systems and could result in more robust or different responses to biased agonists.

Although GLP-1R agonists developed specifically to G protein–directed signaling are yet to be tested in humans, the potential utility of this approach is supported by the recent observation that Tirzepatide, a dual GLP-1R/GIPR agonist peptide currently in late-stage clinical trials (18), and its nonacylated precursor show a significant degree of bias at the GLP-1R in favor of cAMP over β-arrestin recruitment (19, 73). Conflicting reports exist for bias between cAMP and β-arrestin recruitment to the GIPR for Tirzepatide, with one study showing bias in favor of cAMP and another showing no difference (19, 74). Biased agonism at the GCGR is relatively unexplored, except for a recent study of a series of dual GLP-1R/GCGR agonists in which a small response amplitude for β-arrestin-2 recruitment to GCGR hampered bias assessments (75), but should be further explored in the future.

In summary, we demonstrate in this study that GLP-1, GIP, and GCG analogs with a variety of N-terminal substitutions typically show reduced β-arrestin-2 recruitment. In the case of GLP-1 and GIP, this is associated with reduced receptor endocytosis, and this effect can be exploited to increase maximal insulin release *in vitro*. Generation of long-lasting biased incretin mimetics will be required to determine whether this applies *in vivo*.

Experimental procedures

Peptides

All peptides were obtained from Insight Biotechnology and were certified by HPLC to be at least 90% pure.

Cell lines

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Wildtype and dual β-arrestin knockout (37) HEK293 cells were maintained in DMEM, 10% FBS, and 1% penicillin/streptomycin. Monoclonal CHO-K1 cells stably expressing SNAP-GLP-1R or SNAP-GIPR (32) were maintained in Ham's F12 medium, 10% FBS, and 1% penicillin/streptomycin. PathHunter β-arrestin-2 CHO-K1 cells (DiscoverX) stably expressing human GLP-1R, GIPR, or GCGR were maintained in the manufacturer's proprietary culture medium. INS-1 832/3 cells (46), a gift from Professor Christopher Newgard, were maintained in RPMI supplemented with 11 mM glucose, 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1 mM pyruvate, 50 μM β-mercaptoethanol, and 1% penicillin/streptomycin. INS-1 832/3 cells lacking endogenous GLP-1R after deletion by CRISPR-Cas9 (48), a gift from Dr Jacqueline Naylor, AstraZeneca, were maintained similarly. A stable clone of Huh7 hepatoma cells expressing human GCGR was generated from a previously described multiclonal cell population (49) by flow cytometric sorting of cells labeled with FITC-conjugated glucagon, and subsequently maintained in DMEM, 10% FBS, 1% penicillin/streptomycin, and 1 mg/ml G418.

Isolation of primary hepatocytes

Hepatocytes from adult male C57Bl/6J mice were isolated using collagenase perfusion (76). After filtering and washing, cells were plated in 12-well collagen-coated plates at 3 x 10^5 cells/ml, 1 ml per well in attachment medium (M199 with 1% penicillin/streptomycin, 1% BSA, 10% FBS, 100 nM triiodothyronine, 100 nM dexamethasone, and 100 nM insulin). After 5 h, the attachment medium was replaced with serum starvation medium (M199 [Thermo Fisher 31150022] with 1% penicillin/streptomycin, 100 nM dexamethasone, and 10 nM insulin).

Measurement of mini-G and β-arrestin-2 recruitment by nanoBiT complementation

The plasmids for mini-G_s, -G_i, and -G_q, each tagged at the N terminus with the LgBiT tag (21), were a kind gift from Prof Nevin Lambert, Medical College of Georgia. The plasmid for β-arrestin-2 fused at the N terminus to LgBiT was obtained from Promega (plasmid no. CS1603B118). Construction of the GLP-1R-SmBiT plasmid was described previously (55), and the same strategy was used to develop GIPR-SmBiT and GCGR-SmBiT, with cloning in frame at the C terminus of the receptor by substitution of the Tango sequence on a FLAG-tagged GPCR-Tango expression vector (77), a gift from Dr Bryan Roth, University of North Carolina (Addgene # 66295). HEK293T cells in 12-well plates were cotransfected using Lipofectamine 2000 with the following amounts of plasmid DNA: 0.5 μg of GPCR-SmBiT plus 0.5 μg LgBiT-mini-G_s, -G_i, or -G_q; or 0.05 μg each of GPCR-SmBit and LgBit-β-arrestin-2 plus 0.9 μg empty vector DNA (pcDNA3.1). After 24 h, cells were resuspended in Nano-Glo dilution buffer + furimazine

(Promega) diluted 1:50 and seeded in 96-well half area white plates. Baseline luminescence was measured over 5 min using a Flexstation 3 plate reader at 37 °C before addition of agonist or vehicle. After agonist addition, the luminescent signal was serially recorded over 30 min and normalized to well baseline and then to average vehicle-induced signal to establish the agonist effect.

Measurement of β-arrestin-2 activation by intramolecular BRET

HEK293T cells in 6-well plates were cotransfected using Lipofectamine 2000 with the following amounts of plasmid DNA: 0.5 μg SNAP-GLP-1R, SNAP-GIPR, or SNAP-GCGR (all from Cisbio), 0.5 μg Nluc-4myc-β-arrestin-2-CyOFP1 (28), and 1 μg pcDNA3.1. After 24 h, cells were resuspended in Nano-Glo dilution buffer + furimazine (1:50) and seeded in 96-well half area white plates. Baseline luminescence was measured at 460 and 575 nm over 5 min using a Flexstation 3 plate reader at 37 °C before the addition of agonist or vehicle. After agonist addition, luminescent signals at the same wavelengths were serially recorded over 15 min. The BRET ratio (575/460) was calculated at each time point, normalized to well baseline and then to average vehicle-induced signal to establish the agonist-induced BRET effect.

Measurement of receptor internalization by DERET

DERET (8) was used to monitor agonist-induced receptor internalization in HEK293T cells transiently transfected for 24 h with SNAP-tagged receptors (2 μg plasmid DNA per well of 6-well plate) or in monoclonal CHO-K1 cells stably expressing SNAP-GLP-1R or SNAP-GIPR. Labeling was performed using the time-resolved Förster resonance energy transfer SNAP-probe Lumi4-Tb (Cisbio) at 40 nM for 60 min at room temperature, either in suspension (for HEK293T) or with adherent cells (for CHO-K1). After washing three times, fluorescein (24 μM in HBSS) was added to cells in opaque bottom white plates, and baseline signal was read for 10 min using a Flexstation 3 plate reader (λ_{ex} 340 nm, λ_{em} 520 and 620 nm, delay 400 μs, integration 1500 μs) at 37 °C. Agonists, prepared in 24 μM fluorescein, were added, and signal was sequentially monitored. Receptor endocytosis leads to reduced contact of Lumi4-Tb with extracellular fluorescein, and a reduction in signal at 520 nm with an increase at 620 nm. After first subtracting values from wells containing fluorescein only, internalization was expressed ratiometrically as signal obtained at 620 nm divided by that obtained at 520 nm.

Measurement of receptor internalization using a cleavable SNAP-labeling probe

The assay was adapted from a previous description (17). HEK293T or wildtype/dual β-arrestin knockout HEK293 cells were seeded in black, clear bottom, plates coated with 0.1% poly-D-lysine, and assayed 24 h after transfection with SNAP-tagged GLP-1R, GIPR, or GCGR plasmid DNA (0.1 μg per well). Cells were labeled with the cleavable SNAP-tag probe BG-S-S-649 (featuring the DY-649 fluorophore, a gift from New England Biolabs) in complete medium for 30 min at room temperature. After washing, fresh medium ± agonist was added, with agonists applied in reverse time order in the case of time-course experiments. At the end of the incubation, the medium was removed and wells were treated for 10 min at 4 °C with Mesna (100 mM, in alkaline TNE buffer, pH 8.6) to remove BG-S-S-649 bound to residual surface receptor without affecting the internalized receptor population, or with alkaline TNE buffer alone. After washing, cells were imaged using an automated Nikon Ti2 widefield microscope with LED light source (CoolLED) and a 0.75 numerical aperture 20X air objective, assisted by custom-written high-content analysis software (http://www.imperial.ac.uk/a-z-research/photonics/research/biophotonics/instruments–software/high-content-analysis/, accessed February 24, 2020) implemented in Micro-Manager (78). A minimum of four epifluorescence and matching transmitted phase contrast images per well were acquired. Average internalized receptor across the imaged cell population was quantified using Fiji as follows: (1) phase contrast images were processed using PHANTAST (79) to segment cell-containing regions from the background; (2) illumination correction of fluorescence images was performed using BaSiC (80); (3) fluorescence intensity was quantified for cell-containing regions. Agonist-mediated internalization was determined by comparing the mean signal for each condition normalized to signal from wells not treated with Mesna, after first subtracting nonspecific fluorescence determined from wells treated with Mesna but no agonist.

Visualization of receptor redistribution

HEK293T cells seeded on 0.1% poly-D-lysine-coated coverslips were transiently transfected for 24 h with SNAP-GLP-1R, SNAP-GIPR, or SNAP-GCGR (0.5 μg per well of 24-well plate). Surface SNAP-tag labeling was performed using SNAP-Surface-549 (1 μM) for 30 min at 37 °C. After washing, cells were stimulated ±100 nM agonist for 30 min at 37 °C, followed by fixation with 2% paraformaldehyde. Coverslips were mounted using Diamond Prolong antifade with DAPI and imaged using a 1.45 numerical aperture 100X oil immersion objective, with z-stacks acquired throughout the cell volume with a step size of 0.2 μm. Deconvolution was performed using Deconvolutionlab2 (81), and a maximum intensity projection from 10 consecutive z-planes was constructed to generate the final images.

Cyclic AMP assays

All experiments were performed at 37 °C. Wildtype and dual β-arrestin knockout HEK293 cells: 24 h after transient transfection with SNAP-GLP-1R, SNAP-GIPR, or SNAP-GCGR (1 μg per well of 12-well plate), cells were resuspended in serum-free Ham's F12 medium and stimulated with the indicated agonist for 10 or 60 min without phosphodiesterase inhibitors. Forskolin (10 μM) was used as a control. cAMP was quantified by HTRF (cAMP Dynamic 2, Cisbio), and responses were normalized to that of forskolin. PathHunter CHO-K1 cells: Cells were resuspended in serum-free

Ham's F12 medium and treated with the indicated agonist, without phosphodiesterase inhibitors, for 30 min, followed by application of detection reagents for determination of cAMP by HTRF. β-Arrestin-2 recruitment responses were measured in parallel. Huh7-GCGR cells were treated with the indicated concentration of agonist without phosphodiesterase inhibitors before lysis. Three- or 4-parameter curve fitting was performed using Prism 8.0 (GraphPad Software).

Measurement of PKA activation

After cotransfection for 36 h with plasmid DNA encoding the relevant SNAP-tagged receptor (1 μg) and AKAR4-NES (1 μg; a gift from Dr Jin Zhang, Addgene plasmid #647270), wildtype or dual β-arrestin knockout HEK293 cells were suspended in HBSS in black 96-well plates. After a 10-min baseline measurement, compounds were added and fluorescence was measured sequentially using a Flexstation 3 plate reader (λ_{ex} = 440 nm, λ_{em} = 485 and 535 nm). After blank well subtraction, signals were expressed ratiometrically and agonist-induced changes calculated relative to individual well baseline. Curve fitting was performed to determine EC_{50} from the overall signal AUC.

Measurement of ERK1/2 phosphorylation

Twenty-four hours after transient transfection with SNAP-GLP-1R, SNAP-GIPR, or SNAP-GCGR (0.1 μg per well of 96-well plate), wildtype or dual β-arrestin knockout HEK293 cells were stimulated in reverse time order with the indicated ligand (100 nM) in serum-free Ham's F12 medium. ERK1/2 phosphorylation was determined by HTRF (Cisbio Phospho-ERK [Thr202/Tyr204] cellular kit) from cell lysates prepared using the manufacturer's lysis buffer with phosphatase/protease inhibitors. Ligand-stimulated HTRF ratios were normalized for each experiment as a fold change of the HTRF ratio from unstimulated cells.

Measurement of β-arrestin recruitment by enzyme fragment complementation

β-Arrestin-2 recruitment was determined by enzyme fragment complementation using the PathHunter system (DiscoverX). CHO-K1 cells expressing GLP-1R, GIPR, or GCGR were treated with the indicated concentrations of agonist for 30 min at 37 °C before the addition of detection reagents and read by luminescence.

Gene expression analysis

RNA was harvested from INS-1 832/3 and Huh7 cells using the Cells-to-CT kit (Themo Fisher). TaqMan probes were used to detect expression of *Glp1r* (Rn00562406_m1), *Gipr* (Rn00562325_m1), *Gcgr* (Rn00597162_g1), *G6pc* (Rn00689876_m1), and endogenous control gene 18S (Hs99999901_s1) by quantitative PCR.

Insulin secretion

Insulin secretion from INS-1 832/3 cells (46) was assayed after a prior overnight period of exposure to low-glucose (3 mM) complete medium. Cells were detached from flasks using 0.02% EDTA and added in suspension to plates containing the indicated agonists, prepared in RPMI containing 2% FBS and 11 mM glucose, for 16 h. The supernatant insulin concentration was determined by HTRF (High Range Insulin kit, Cisbio). Results were normalized to those obtained with 11 mM glucose but no additional agonist. Three-parameter fitting was performed using Prism 8.0.

Data analysis and statistics

All analyses were performed using Prism 8.0. For bias calculations, to reduce the contribution of interassay variability, cAMP and β-arrestin-2 assays were performed concurrently, with the same incubation time of 30 min to avoid artefactual bias resulting from different activation kinetics of each pathway (65); bias was determined by calculating transduction coefficients (43, 82); here, owing to the matched design of our experiments, we calculated $\Delta\Delta\log(\tau/K_A)$ on a per-assay basis by normalizing the $\log(\tau/K_A)$ of each ligand to the relevant endogenous ligand (GLP-1, GIP, or GCG, to generate a $\Delta\log[\tau/K_A]$ value) and then to the reference pathway (cAMP). For experiments with a matched design, paired two-tailed *t* tests or randomized block ANOVAs were performed. Specific statistical tests are indicated in the figure legends. Statistical significance was inferred when $p < 0.05$. To determine statistical significance for biased agonism, 95% confidence intervals were calculated; bias *versus* the reference endogenous ligand was considered statistically significant when this confidence interval did not cross zero, as previously recommended (82).

Data availability

Any additional data supporting the analyses in the manuscript is available from B. J. on reasonable request.

Author contributions—B. J., S. R. B., and A. T. conceived and designed the study. B. J., E. R. M., Z. F., and P. P. performed and analyzed experiments. I. R. C., A. O., R. J., and A. I. provided novel reagents. S. K., F. G., C. D., and P. M. W. F. developed HCA instrumentation and analysis tools. Funding for the study was acquired by B. J., A. T., S. R. B., G. A. R., and T. T. B. J. wrote the draft manuscript. All authors made manuscript revisions and approved the final version.

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Abbreviations—The abbreviations used are: DERET, diffusion-enhanced resonance energy transfer; DPP-4, dipeptidyl dipeptidase-4; GCG, glucagon; GCGR, glucagon receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; GPCR, G protein–coupled receptors; MAPK, mitogen-activated protein kinases; PKA, protein kinase A; T2D, type 2 diabetes.

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